REMARKS

Claim 1 has been amended to limit the antigens to peptides, polypeptides, proteins or nucleic acids encoding them as set forth in original claim 2. Claim 1 has also been amended to specify that an immunogenic potentiating effective amount of the vaccine carrier is present in the pharmaceutical composition. Support for this amendment can be found, for example, at page 5, lines 1-3, page 6, lines 14-17, page 7, lines 14-17 and page 8, line 27 - page 9, line 25 of the specification, as well as the numerous examples. Claim 1 has further been amended to specify that the pharmaceutical composition stimulates both humoral and cellular responses against the antigen. Support for this limitation can be found, for example, at page 7, lines 7-10 of the specification, as well as the numerous examples.

Claim 2 has been canceled in view of the amendments to claim 1.

Claim 3 has been amended in view of the cancellation of claim 2.

Claim 8 has been amended to include N-acetyl variations of the gangliosides. Support for this amendment can be found at page 7, lines 17-19 of the specification.

Claim 28 has been amended to restrict it to N-acetyl GM3 as supported by the amendment to claim 8.

Claim 29 has been added to be directed to the elected species of the growth factor receptor HER-1.

Applicants submit that the above amendments do not add any new matter, and their entry is requested.

In the present Office Action, the Examiner maintained the rejection of claims 1-10, 27 and 28 under 35 U.S.C. § 103 (a) as being obvious over Rodriguez et al. (US 5,788,985) in view of Hammonds et al. (US 4,857,637) and Udayachander et al. (*Human Antibodies* **8**:60-64, 1997). The Examiner repeated the rejection as set forth in the first Office Action. In essence, the Examiner cites Rodriguez et al. for its disclosure of the OMPC of *Neisseria meningitides* into which N-glycoyl GM3 has been incorporated (i.e., VSSPs) and its use for treating breast cancer. The Examiner cites Hammonds et al. for its disclosure of using EGFR (i.e., HER-1) as an antigen to immunize animals

and for its disclsoure that EGFR is overexpressed in malignant cells and thus is a desirable target for therapy. Hammonds et al. also discloses the use of an adjuvant for immunization with growth factor receptors. Finally, the Examiner cites Udayachander et al. for its disclosure that many malignancies, such as breast cancer overexpress EGFR and that EGFR is a target for therapy. In view of these teachings in the art, the Examiner concludes that it would be *prima facie* obvious to combine the two treatments of Rodriguez et al. and Hammonds et al. into a single treatment for breast cancer because a skilled artisan would be motivated to use both compositions in combination in a method for treating a malignant tumor that overexpresses the two antigens, such as breast cancer. However, as demonstrated below, it is submitted that the Examiner is in error in this rejection.

In reply to the first Office Action, Applicants argued that that although Rodriguez et al. discloses VSSPs, there is no disclosure in Rodriguez et al. that the VSSPs can be utilized to potentiate the immunogenicity of low immunogenic antigens, such as growth factor receptors. In addition, there is no disclosure in any of the secondary references that the VSSPs of Rodriguez et al. could potentiate the immunogenicity of low immunogenic antigens. Because there is no suggestion in Rodriguez et al. that the immunogenicity of low immunogenic antigens, such as growth factor receptors, could be potentiated, there is no motivation to combine the references as proposed by the Examiner. There is no suggestion in either of the secondary references that the immunogenicity of the disclosed antigens should be potentiated. In the absence of any teaching in the primary or secondary references concerning potentiation of the immunogenicity of growth factor receptors, there is no motivation or suggestion in the cited art to combine these references in the manner proposed by the Examiner. Interestingly, the Examiner simply repeated the prior rejections and did not address where the motivation to combine could be found in the cited references or within the general skill in the art, particularly in light of the lack of any teaching in the cited prior art of the adjuvant property of the VSSPs. Thus, Applicants submit that the cited prior art does not render the subject matter of claims 1-10, 27 and 28 obvious.

More specifically, Rodriguez et al. discloses a composition for eliciting an immune response against N-glycolylated gangliosides. The immune response is elicited by incorporating the N-glycolylated gangliosides in the outer membrane protein complex of Neisseria meningitidis resulting

in the production of VSSPs. Administration of the VSSPs containing the N-glycolylated gangliosides elicits an immune response against the N-glycolylated gangliosides. The vaccine composition of Rodriguez et al. comprises the VSSPs as the antigen. As Applicants previously argued, there is no disclosure in Rodriguez et al. that the VSSPs can be used as a carrier, i.e., an adjuvant, to potentiate, i.e., increase, the immunogenicity of low immunogenic antigens that are peptides, polypeptides, proteins or nucleic acids that encode them. The combination of Rodriguez et al and Hammonds et al. is simply the combination of two antigens in a vaccine composition. There is no disclosure in either of these references alone or together that the VSSPs can act as a carrier, i.e. an adjuvant, to potentiate the immune response against a low immunogenic peptide antigen. Because there is no such disclosure in these references, there is no disclosure that an immunogenic potentiating effective amount of the VSSPs should be used in the composition. Furthermore, there is no disclosure in these references alone or together that the composition stimulates both a humoral and cellular response against the low immunogenic peptide antigen. Thus, Applicants submit that the Examiner's combination of references does not render the claimed subject matter obvious.

As Applicants previously argued, they have surprisingly found that the claimed pharmaceutical composition of the present invention confers immunogenicity to peptides, polypeptides, proteins and their corresponding DNA sequences (which are molecules completely different from the immunological point of view) by mixing them with VSSPs, such as described by Rodriguez et al. Furthermore, this technological solution allows the use of the whole structure of the growth factor receptors, thereby solving the immune dominance genetic restriction. As shown throughout the present application, the VSSPs are used as an adjuvant (carrier) that potentiates the immunogenicity of peptide antigens. The use of VSSPs as an adjuvant (carrier) is not disclosed in Rodriguez et al. or in the secondary references.

As a difference from the cited prior art, the claimed pharmaceutical compositions of the present invention shows surprising immunological properties such as a dramatic ability to cause dendritic cells maturation and restoring immune-suppressed patients. Moreover, the claimed compositions of the present invention have the capacity of stimulating both humoral and cellular

responses against a particular low immunogenic antigen. Applicants have amended the claims to include this latter limitation. This limitation is not disclosed or suggested in the prior art references.

Taking into account the very well known fact that the immune response generated by proteins is quite different from that generated by carbohydrates, this important feature of claimed compositions described of the present invention has been neither suggested nor anticipated by Rodriguez et al. or any other prior art. The claims have been limited the low immunogenic antigens being peptides, polypeptides, proteins or nucleic acids encoding them. The claims do not encompass carbohydrate antigens.

As previously argue, two main features distinguish the present invention from the cited prior art and from the state of art:

- An innovative aspect of the present invention is the rather small size of the particles in VSSP. It is very well known in the art that the outer membrane protein complex of *Neisseria* can be used for obtaining immunogens. However, the present invention relates to Applicants' finding that these nanoparticles have an adjuvant capacity in which the ganglioside plays a crucial role in substantially increasing the adjuvant capacity of the protein complex. The claims have been amended to specify that an immunogenic potentiating effective amount of the VSSPs is included in the composition.
- The other innovative aspect is relates to the biological functions of the ganglioside. Applicants note that gangliosides other than GM3 can be used in the present invention. The GM3 ganglioside is one of the most powerful immune suppressants. However, Applicants have found that the side effect targeting of GM3 in VSSP renders pharmaceutical compositions with the capacity of stimulating both humoral and cell responses, particularly in individuals with a deeply depressed immune system, like patients with cancer or AIDS. In these individuals, the use of VSSP is reasonably a superior adjuvant solution than using just the natural outer membrane protein complex alone. In addition and particularly relevant for vaccine compositions including VSSP and EGF-R related polypeptides is the fact that GM3 co-modulates the signal transduction function of the receptor, once triggered by growth factors. Thus, the

VSSP/EGF-R vaccine of the present invention is the only possible technical solution which resembles the real scenario of natural biological interactions of these molecules in the cells.

The Examiner cites Hammonds et al. for its disclosure of using EGF-R as an antigen to immunize animals. As is known to those skilled in the art, "enhancing immunogenicity of poorly immunogenic antigens" is a purpose that is achieved by using immunity-improving agents. Although Applicants agree with this declaration, Applicants nevertheless note that it is a broad concept which enunciates a complex problem. This complex problem has triggered the development of vaccine field. Moreover, this concept expresses a desire which itself is unpatentable, but for which the practical solutions are patentable. The manner in which the immune system responds against any molecule depends on the particular properties of each molecule. The concept is so broad and hard to understand such that it is not predictable. The immunogenicity of a given antigen or type of antigen and the potentiation of a given antigen or type of antigen requires particular solution for each antigen or type of antigen. Any person skilled in the art knows many examples which testify that the technical solution for one kind of molecule doesn't work with another.

In fact, a procedure to enhance the immunogenicity of a particular antigen or type of antigen is not obvious from the preceding procedures described in the art, even for molecules with the same nature. For example, the tetanus toxoid vaccine was described many years ago. It could be considered that mixing any protein with aluminium hydroxide would be the universal solution for increasing the immunogenicity of proteins. However, it has been thoroughly demonstrated that whereas said procedure enhanced the immunogenicity of the tetanus toxoid antigen, it failed for many other proteins. Thus, Applicants submit that the subject matter of the present invention, i.e., the potentiation of the immunogenicity of low immunogenic peptide antigens, is very complex, that individual practical solutions are needed and that none of the solutions are obvious from the previous ones. This fact is especially relevant when comparing the immunogenicity between proteins, carbohydrates and DNA for which the immune system has completely different performance. As previously noted, the present claims are limited to peptide antigens, polypeptide antigens, protein antigens or nucleic acids encoding them.

Applicants submit that that stimulating or increasing the immunogenicity of any molecule is a trial and error procedure, which requires the researcher's resourcefulness in order to find the exact composition for inducing the highest immune response as well as the kind of the response, such as an IgM or IgG or T cell immune response or the like. As previously noted, the present claims specify that the pharmaceutical composition stimulates both a humoral response and a cellular response. This property is not suggested by the cited prior art.

Applicants note that the aim of Hammonds et al. (US 4,857,637) is to induce **an autoimmune** response to achieve a desired therapeutic effect. Furthermore, novel compositions provided in Hammonds et al. include conjugates of an immunogenic substance with a derivative or polypeptide fragment of a receptor to raise an **antibody response** which is consistently and substantially working either as ligand agonist or antagonist (see column 2, lines 63). **Applicants consider this statement as an intended use because any skilled person knows that the kind of immune response can't not be predicted when an animal is inoculated with an immunogen.** Moreover, the polyclonal antibody response raised upon immunization is heterogeneous. This response includes a substantial degree of both ligand agonist and antagonist effect. To choose the fragments or derivates to be substantially devoid of immunologically-recognized epitopes which will induce a polyclonal response in which antibody are present that have the undesired activity is also a desire more than a fact. To select such fragments or derivates is not a straightforward problem. It would require a trial and error procedure and the inventiveness of the researchers as well as a lot of work.

In addition, Applicants note that in another embodiment, Hammonds et al. requires that **the polypeptides should be heterologous or foreign** to the animal species being immunized. Finally, Hammonds et al. states that in all cases **the immunizations are to be conducted in accordance with standard methods for raising antibodies** against proteins or polypeptides, e.g., by the use of adjutants and/or conjugating proteins with immunogens such as keyhole limpet hameocyanin and others.

In contrast, **the aim of the present invention is not merely to teach a pharmaceutical composition comprising the epidermal growth factor receptor**, or any other growth factor as an

antigen to immunize animals against the growth factor. The present invention discloses **a composition with adjuvant properties**, which stimulates or increases the immunogenicity of **proteins and fragments thereof and the nucleic acids encoding such proteins**, even for those proteins and fragments for which the standard methods have been unsuccessful (the references hereinafter discussed provide evidence about this statement). This feature of the VSSPs has not been anticipated or suggested by any of the prior art.

Moreover, the present invention intended not only an antibody response by means of the agonist and antagonist of the growth factor receptor but also to stimulate the whole immune system of the animal or human being, i.e., both a humoral and a cellular response as set forth in the claims. **The present invention provides evidences regarding the relative superiority of VSSP as adjuvant for different antigen variants, including a model of the HER1 extra cellular domain protein**. The numerous examples in the present application provide evidence of the surprising results obtained with the present invention. These results have been confirmed by the following peer-reviewed papers.

1. Maturation of dendritic cells as a response to the treatment with the VSSP preparation. Mesa, C. et al., "Very small sized proteoliposomes derived from *Neisseria meningitides*: an effective adjuvant for Th1 induction and dendritic cell activation," *Vaccine* **22**:3045-3052, 2004 (copy attached); Mesa, C. et al., "Very small size proteoliposomes derived from Neisseria meningitidis: an effective adjuvant for dendritic cell activation," *Vaccine* **26 Suppl 2**:S42-S43, 2006a (copy epub attached).

2. Induction of a specific humoral immune response associated to the administration of the vaccine composition. Table 3 of the present application shows that the humoral response was significant higher in those animals immunized with the VSSP preparation. Mesa, C. et al. (2004); Sanchez-Ramirez, B. et al., "Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant," *Int J Cancer* **119**:2190-2199, 2006 (copy attached).

3. Induction of a specific proliferative and cytotoxic cell response when immunizing with the VSSP preparation. Mesa, C. et al., 2004; Torrens, I. et al., "Immunotherapy with CTL peptide and

VSSP eradicated established human papillomavirus (HPV) type 16 E7-espressing tumors," *Vaccine* **23**:5768-5774, 2005 (copy attached).

4. The relative expression levels of some T cells markers in the peripheral mononuclear cells of the same patients returned to regular levels after receiving VSSP preparation. It reinforced two main characteristic of the claimed invention.

- The VSSP preparation stimulates the whole immune system not only the antibody response.
- The VSSP preparation rendered a safety adjuvant for humans avoiding the adverse effects of most of the oily adjutants.

In addition to the examples of the present application, Applicants have also reported the effect of the VSSP preparation in the following peer-reviewed papers.

1. **Adjuvant for proteins**: it has been demonstrated for OVA (chicken albumin) and the murine extra cellular domain of the EGF receptor (mECD-EGF-R). (Mesa, C. et al., 2004; Mesa, C. et al., "Very small sized proteoliposomes derived from *Neisseria meningitides*: an effective adjuvant for generation of CTL responses to peptide and protein antigens," *Vaccine* **24**:2692-2699, 2006b (copy of epub attached); Sanchez-Ramirez, B. et al., 2006.

2. **Adjuvant for peptides**: It has been demonstrated for OVA derived peptides and for peptides derived from the Human Papilloma Virus protein E7. Mesa, C. et al., 2006b; Torrens, I. et al., 2005.

3. **Adjuvant for cellular vaccines**: it has been tested using two tumour models (CT26 is a colon cancer and F3II is a breast tumour). Mesa, C. et al., 2006b.

Applicants submit that the objective evidence provided in the numerous examples in the present application as well as the objective evidence presented in the attached peer-reviewed papers clearly establishes the surprising and unexpected adjuvant property of the claimed VSSPs, i.e., VSSPs derived from the Outer Membrane Protein Complex (OMPC) of *Neisseria meningitidis* wherein gangliosides have been incorporated into the OMPC, and the corresponding surprising and unexpected property that the VSSPs are able to potentiate the immunogenicity of low immunogenic antigens that are peptides, polypeptides, proteins or nucleic acids that encode them. Furthermore,

this objective evidence further demonstrates the surprising and unexpected property that the pharmaceutical composition containing the specified low immunogenic antigens and the VSSPs stimulates both the humoral and cellular immune responses. One of the cellular response shown by this objective evidence is the activation and maturation of dendritic cells. Neither of these properties is taught by or suggested by the cited prior art references alone or in combination. Furthermore, the cited prior art references do not suggested a pharmaceutical composition that comprises the specified low immunogenic antigens (i.e., peptides, polypeptides, proteins or nucleic acids encoding them) and an immunogenic potentiating effective amount of VSSPs. Thus, Applicants submit that the cited prior art does not render the claimed invention obvious.

In addition, Applicants note that claim 7 includes N-acetylated variants of GM1 and GM3 and that claim 28 is directed to the gangliosides being N-acetylated GM3. With respect to these claims, Applicants note that Rodriguez et al. discloses VSSPs containing N-glycolylated gangliosides, particularly GM3. The expression of certain gangliosides in mammalian tissues is species restricted. Thus, N-glycolylated gangliosides (including GM3) are present in most species (mice, rats, dogs, horses, pigs, etc) but not humans and chickens. In contrast, the ganglioside of claim 28 is N-acetylated GM3. N-acetylated GM3 is the more abundant ganglioside in extra neural tissues. This difference is important for the present application because a skilled person knows that the intensity of the immune response to any molecule will inversely depend on their degree of expression in normal tissues. The results of immunizing human beings with these two gangliosides are quite different. Whereas N-glycolylated gangliosides acts as "foreign" antigens, raising a potent immune response in humans, N-acetylated gangliosides are "self" antigens and much less immunogenic. For adjuvants a low immunogenicity of their components is desirable, allowing that the vaccine induced immune response can be concentrated in the accompanying antigen. Thus, Applicants submit that compositions containing VSSPs containing N-acetylated gangliosides is not obvious from the cited references.

In view of the above remarks, it is submitted that the present invention is not obvious over Rodriguez in view of Hammond et al. and Udayachander et al. Withdrawal of this rejection is requested.

In the present Office Action, the Examiner maintained the rejection of claim 11 under 35 U.S.C. § 103 (a) as being obvious over Rodriguez et al. in view of Hammonds et al. and Udayachander et al. and in further view of Carr et al. (*Melanoma Research* **11**:219-227, 2001). The Examiner repeated the rejection as set forth in the first Office Action. Carr et al., which was published in June 2001, is cited for its disclosure of Montanide ISA51. However, as demonstrated above, it is submitted that the Examiner is in error in this rejection.

Specifically, the combination of Rodriguez et al., Hammonds et al. and Udayachander et al. does not render obvious the subject matter of claims 1-10, 27 and 28 as detailed above. Because the tertiary reference, Carr et al., does not supply any of the deficiencies of the cited primary and secondary references, it cannot render the subject matter of claim 11 obvious. Furthermore, Carr et al. described the effect of the ganglioside vaccine on the B16 mouse melanoma experimental model. The vaccine composition used by Carr et al. is the same vaccine composition described in Rodriguez et al., therefore the same comments made above with respect to Rodriguez et al. also apply to Carr et al. Thus, Applicants submit that the subject matter of claim 11 is not obvious from Rodriguez et al., Hammonds et al. and Udayachander et al. further in view of Carr et al.

In view of the above remarks, it is submitted that the present invention is not obvious over Rodriguez in view of Hammond et al. and Udayachander et al. and further in view of Carr et al. Withdrawal of this rejection is requested.

Applicants submit that the presently amended claims include limitations that the Examiner noted were not present in the claims to which arguments were directed in the previous response which absence from the claims was raised for the first time in the final rejection. In addition, the claims include a limitation to the amount of VSSPs present to clearly delineate the present invention from the Examiner's proposed combination of a prior art composition. None of the prior art suggests the adjuvant property of VSSPs and thus does not suggest using an immunogenic-potentiating effective amount of VSSPs in a pharmaceutical composition. Furthermore, Applicants submit that the present application and the peer-reviewed papers discussed herein and attached hereto provide objective evidence of the adjuvant property of the VSSPs and the ability of the claimed composition

to stimulate both humoral and cellular responses to the claimed low immunogenic antigens. Thus, Applicants submit that the claimed subject matter is not obvious from the cited prior art.

In view of the above amendments and remarks, it is believed that the present claims satisfy the provisions of the patent statutes and are patentable over the cited prior art. Reconsideration of the application and early notice of allowance are requested. The Examiner is invited to telephone the undersigned to expedite the prosecution of the application.

Respectfully submitted,

ROTHWELL, FIGG, ERNST & MANBECK, p.c.

By _____

Jeffrey L. Innen
Registration No. 28,957
Attorney for Applicants
1425 K Street, N.W., Suite 800
Washington, D.C. 20005
Telephone No.: (202) 783-6040
Facsimile No.: (202) 783-6031

ATTACHMENTS:

Mesa, C. et al., "Very small sized proteoliposomes derived from *Neisseria meningitides*: an effective adjuvant for Th1 induction and dendritic cell activation," *Vaccine* **22**:3045-3052, 2004.

Mesa, C. et al., "Very small size proteoliposomes derived from Neisseria meningitidis: an effective adjuvant for dendritic cell activation," *Vaccine* **26 Suppl 2**:S42-S43, 2006a (epub).

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Mesa, C. et al., "Very small sized proteoliposomes derived from *Neisseria meningitides*: an effective adjuvant for generation of CTL responses to peptide and protein antigens," *Vaccine* **24**:2692-2699, 2006b (epub).

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Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for Th1 induction and dendritic cell activation

Circe Mesa [a,*], Joel de León [a], Kevin Rigley [b], Luis E. Fernández [a]

[a] *Vaccine's Department, Centre of Molecular Immunology, 216 esq 15, Atabey, Playa, CP 16040 Havana, Cuba*
[b] *Dendritic Cell Group, Edward Jenner Institute for Vaccine Research, Compton, Newbury RG20 7NN, UK*

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Abstract

Recent findings about pathogens and innate immune system interactions have opened new opportunities for adjuvants designs. We have elaborated a new approach, in which gangliosides are incorporated into the outer membrane complex of *Neisseria meningitidis* (Nm) to form very small size proteoliposomes (VSSP). VSSP, used as monotherapy, demonstrated a unique ability to render immunogenic highly tolerated gangliosides. These results drove our attention to the immunopotentiating properties of VSSP. Here, we examined the VSSP adjuvant effect on the humoral and cellular responses, dendritic cell (DC) activation, and differentiation of Th cells. Also, the role of LPS in VSSP effect was dissected. This study reveals that VSSP is a potent adjuvant for dendritic cells activation and Th1 differentiation.

Keywords: Dendritic cells; Th1/Th2 cells; *Neisseria meningitidis*

1. Introduction

Adjuvants are important components of vaccine formulations, acting as enhancers of antigens' immunogenicity. However, traditional adjuvants sometimes do not stimulate protective immunity because they fail to fully mobilize the appropriate responses. Moreover, many of them promote serious adverse side effects and are therefore, not suitable for human use.

Emerging theories and knowledge about the immune system regulation have strongly influenced in adjuvant development. New pathogen-related molecules have been recently identified as "danger" signals [1], switching on the innate immune system, basically by dendritic cells (DC) maturation. It has also been proposed that the pattern recognition receptors expressed on DC interact with their ligands to subsequently condition either a Th1 or Th2 response [2,3].

Neisseria meningitidis (Nm) interacts with DC inducing strong secretion of proinflammatory cytokines TNF-α, IL-6, IL-8 and IL-12 production [4,5]. This bacteria spontaneously sheds natural outer membrane vesicles (nOMV) [6], which are considered potential adjuvants since many of their components are B-cell mitogens and others, such as the major antigen PorA, augment the allostimulatory properties of treated DC [7–9]. In this respect, we have described new cancer vaccines formulations based on nOMV adjuvanticity properties and the hydrophobic incorporation of NAcGM3, NGcGM3 or NAcGM1 gangliosides to form very small size proteoliposomes (VSSP) [10]. Immunization of mice with VSSP emulsified in an oily adjuvant consistently induced highly specific IgM and IgG antibodies against the incorporated ganglioside. Moreover, vaccination with $VSSP^{NAcGM3}$ increased the overall survival of mice challenged with the NAcGM3 positive melanoma B16 tumor [11]. Phase I trials of VSSP in melanoma and breast cancer patients have already demonstrated the safety and immunogenicity of these preparations [12].

This peculiar ability of VSSP to render immunogenic ubiquitous gangliosides, suggested strong immunopotentiatory properties for VSSP, in which incorporated gangliosides could play a role in its physicochemical characteristics. Also, a potential biological role of the ganglioside in VSSP should not be neglected and is currently under investigation.

In the present study, we established that VSSP promoted strong antibody and cellular responses to OVA, either emulsified or not. The adjuvanticity of this formulation is mediated by a proper DC maturation, with the corresponding IL-12p40/p70 production. Experiments with transgenic mice-derived T cells showed that VSSP conditioned a Th1 phenotype on stimulated naïve T cells. As VSSP are es-

* Corresponding author. Tel.: +53-7-271-6810; fax: +53-7-272-0644.
E-mail address: circe@ict.cim.sld.cu (C. Mesa).

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sentially bacterial membranes, we studied the contribution of LPS on VSSP properties. LPS hyporesponsive C3H/HeJ mice-derived DC were properly activated by VSSP.

2. Materials and methods

2.1. Mice

For in vivo experiments Balb/c mice were purchased from the Centre for Laboratory Animal Production (CENPALAB, Havana, Cuba) and maintained in the animal house of the CIM, Havana, Cuba. Balb/c (for in vitro experiments) and DO.11.10 αβ TCR transgenic (Tg) mice were bred and maintained in the SPF unit of the Institute for Animal Health, Compton, Berkshire, UK. C3H/HeJ and C57Bl/6 mice were supplied by Harlan UK, Bicester, Oxon. All animals were between 6 and 12 weeks of age.

2.2. Reagents used for stimulation studies

Dr. Svein Andersen, EJIVR, kindly provided purified lipopolysaccaride (LPS) from *N. meningitidis* strain 44/76. Monophosphoryl lipid-A (MPL-A) from *Escherichia coli* strain F583 was purchased from Sigma Chemical, UK, and the anti-mouse CD40 (1C10) from R&D Systems Ltd., Europe. VSSP [10] is produced and provided by the Centre of Molecular Immunology (Havana, Cuba).

2.3. Immunizations

For humoral response experiments, three groups of 10 Balb/c mice were immunized s.c., three times at 2-week intervals, with 50 μg of OVA (Sigma, San Louis, MO), emulsified by mixing equal volumes of OVA solution with complete Freund's adjuvant (CFA), or OVA + VSSP (120 μg) solution with Montanide ISA 51 (Seppic, France), a special type of incomplete Freund's adjuvant [13]. A non-oily immnunogen was also prepared by mixing 50 μg of OVA with 120 μg of VSSP in Tris–HCl buffer (pH 8.5). Animals were bled 1 week before and after the last immunization. Sera were stored at −20 °C.

For cellular response experiments, one single dose of 100 μg of OVA plus adjuvants were administered s.c. near the base of the tail, to three groups of Balb/c mice (three animals each). One week later, mice were killed and inguinal lymph nodes were removed.

2.4. ELISA assay for OVA-specific antibody production

Solid-phase ELISA was performed using 96-well polystyrene plates (High binding, Costar), coated with 10 μg/ml of OVA (Sigma, San Louis, MO) overnight at 4 °C. Then the diluted serum samples were incubated 2 h at 37 °C. The second antibody, Fc fragment-specific biotinylated rat anti-mouse IgG (Jackson, WestGrove, Pensylvania), was added and after 1 h of incubation the plates were washed, then streptavidine–phosphatase conjugate (Jackson, WestGrove, Pennsylvania) was added and the reaction developed with TMB substrate (PharMingen, San Diego, California, USA) until the addition of H_2SO_4 1N. Absorbance was measured at 405 nm in an ELISA reader (Organon Teknika, Salzburg, Austria).

2.5. Cellular proliferation assay

Inguinal lymph nodes from mice previously immunized with OVA, as described, were smashed and made into single cell suspension in RPMI 1640 (Gibco, UK) with Glutamax I and 25 mM HEPES (Gibco, UK) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. Obtained cells were then cultured in the presence of graded amounts of OVA. Proliferation was monitored by measuring [methyl $-^3$H] TdR (1 μCi per well) (Amersham, UK) incorporation on day 4 of culture. Radioactivity was determined on a Topcount microplate scintillation counter (Wallac, Finland).

2.6. FACS analysis

DC were analyzed for expression of surface molecules by flow cytometry. Cells were stained with either PE- or FITC-conjugated MAb: hamster anti-mouse CD11c (HL3) conjugated to PE, CD40 (HM40-3) FITC, rat anti-mouse MHC class II/I-Ed (2G9), CD80/B7.1 (GL1), CD86/B7.2 (GL1) all conjugated to FITC, and anti-CD4 (RM4-5) conjugated to allophycocyanin (APC) for CD4 T cell sorting. For human experiments mouse antihuman CD86 (IT2.2), HLA-DR (G46-6), CD1a (HI149), CD11c (B-ly6) conjugated to PE and anti-CD40 conjugated to FITC, were used. Intracellular detection of IL-12 was performed using BD Cytofix/Cytoperm™ Plus kit with BD GolgiPlug™ kit following the protocol recommended by the manufacturer and using the APC-conjugated rat anti-mouse IL-12p40/70 (C15.6). Permeabilization kit, antibodies and all of the matching isotype controls were from Pharmingen, Becton Dickinson, UK.

2.7. Mouse bone marrow DC preparation

Bone marrow (bm)-derived DC were prepared as described elsewhere [14]. Briefly, bone marrow cells were cultured in Isocove's Modified Dulbecco's medium (Life Technologies Ltd., Paisley) supplemented with 10 ng/ml of GM-CSF (R&D Systems, Abingdon, Oxon) at approximately 10^6 cells/ml. At day 7, bmDC were further purified (>95%) using magnetic anti-CD11c beads, according to the manufacturer's instructions (Milteny Biotec, Surrey, UK). Following isolation, bmDC were resuspended in fresh media at 10^6 cells/ml with or without additional stimuli.

2.8. Human DC preparation

PBMC were isolated by standard density gradient centrifugation with Histopaque (1.077 g/ml) (Sigma, UK). PBMC were harvested for the subsequent monocyte isolation. $CD14^+$ cells were enriched by high gradient magnetic sorting negative selection using the MidiMACS monocyte isolation kit (Miltenyi Biotech GmBH, Germany) following supplier's recommendations. Isolated $CD14^+$ monocytes were cultured in RPMI (Life Technologies Ltd., Paisley) supplemented with 50 ng/ml of recombinant human (rh) GM-CSF and 1000 U/ml of rh IL-4 (R&D Systems, UK). At day 7 cells were cultured with or without additional stimuli.

2.9. Antigen-specific T cell activation assay

Purified T cells from DO.11.10 mice were used as source of antigen-specific responder cells. The naive cells were first isolated from spleens using anti-mouse CD62L magnetic beads (Milteny Biotec, Germany) and further stained with APC-conjugated CD4 for sorting by MoFlo FACS sorter (Cytomation, Ely, UK). T cells were then cultured with the relevant bmDC and different concentrations of Ovalbumin peptide (323-ISQAVHAAHAEINEAGR-339) (Genosys Europe, Pampisford, Cambridgeshire, UK). Proliferation was monitored by measuring [methyl $-^3$H] TdR (1 μCi per well) (Amersham, UK) incorporation on day 4 of culture. For the cytokine assays, at day 3 of culture, 500 ng/ml ionomycin and 50 ng/ml PMA (Sigma, San Louis, MO) were added to the cells. Supernatants were removed 24 h later and cytokines were evaluated using QuantikineTM Mouse ELISA kits (R&D Systems Ltd., Abingdon, UK), following the protocol recommended by the manufacturer.

2.10. RNase protection assay

Bone marrow DC from C57Bl/6 and C3H/HeJ mice were cultured in the presence of different stimuli for 8 h. The total RNA was extracted using RNeasy Midi kit (Qiagen Ltd., UK). Cytokine transcript levels were determined by RPA using RiboQuantTM kits (Pharmingen, Becton Dickinson, UK) with the probe sets mCK-5 and mCK-2b. The intensities for each band were corrected for background levels and normalized for differences between each sample using the average values for the housekeeper genes GAPDH and L32.

2.11. Statistical analysis

Differences in humoral response between all treatment groups were analyzed by one-way ANOVA and Tukey's test for pairwise comparisons. For cellular response the significance of differences between all experimental groups was analyzed by Kruskall Wallis and Tukey's test for pairwise comparisons. Value were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. VSSP promote antigen-specific humoral and cellular responses

We have previously shown, in the ganglioside model, that immunization of mice with VSSP/Montanide ISA 51 induced high levels of anti-ganglioside-specific IgG isotypes [10]. However, the VSSP adjuvant effect for an accompanying protein has never been assessed, nor the mandatory need of emulsions. To address this point, groups of Balb/c mice were injected s.c. with OVA in the presence of VSSP, either emulsified or not in Montanide ISA 51, the same preparation with CFA, as a reference, was used. As shown in Fig. 1a, emulsified VSSP induced similar anti-OVA IgG levels compared with CFA (Tukey's test, $P = 0.151$). Moreover, VSSP alone produced large quantities of IgG, reaching antibody titters up to $1/6 \times 10^6$. Anti-OVA cellular

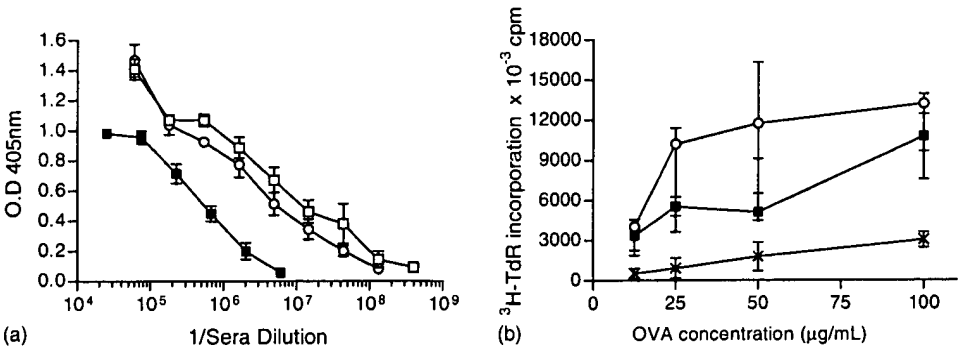

Fig. 1. Antigen-specific humoral and cellular responses induced by immunization of Balb/c mice with OVA/VSSP. (a) Humoral response. Three groups of 10 mice were injected s.c. with OVA/CFA (○), OVA/VSSP/Montanide ISA 51 (□) or OVA/VSSP (■). Sera were collected from mice 7 days after the last injection and assayed by ELISA for anti-OVA total IgG. Data are represented as mean ± S.D. (b) Cellular response. Cells from drained lymph nodes were obtained 1 week after a single dose of OVA/VSSP (■) or OVA/CFA (○) or OVA/PBS (×), to perform a cellular proliferation assay. Median and ranges, corresponding to three mice from each group of immunization, are shown.

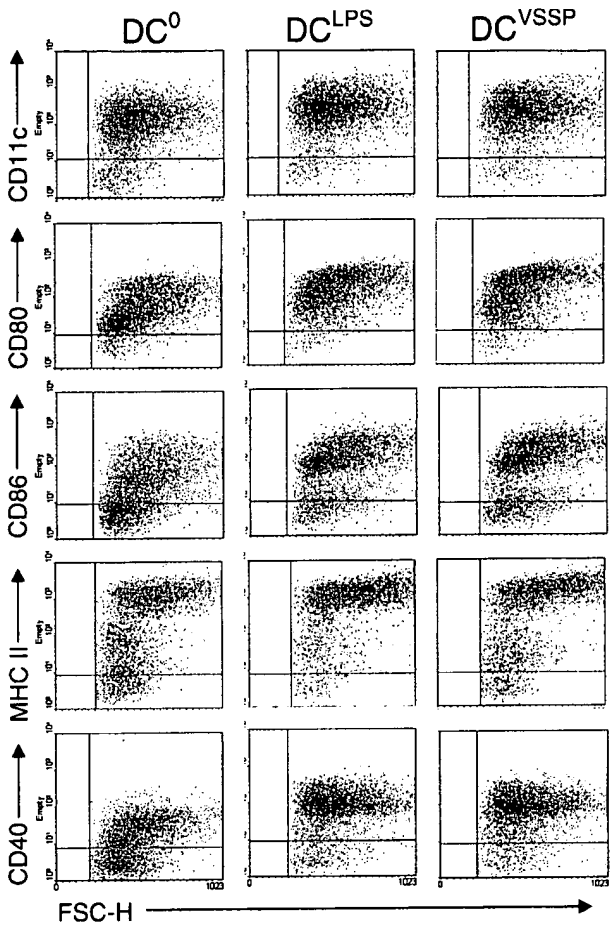

Fig. 2. Dendritic cell maturation induced by VSSP. CD11c$^+$ bmDC were cultured for 24 h with VSSP, LPS or medium and expression of CD11c, MHC II, CD80, CD86 and CD40 was examined by flow cytometry. Data are presented in dot plots from a single experiment representative of several others.

response elicited by non-emulsified VSSP was also measured in a proliferation assay. Different amounts of OVA were added to inguinal lymph nodes cells obtained from mice immunized with a single OVA-VSSP dose. CFA was again used as a reference. Noteworthy, VSSP was able to induce an OVA-specific proliferative response similar to that induced by CFA (Tukey's test, $P = 0.185$) (Fig. 1b).

3.2. VSSP induce maturation of bmDC

Dendritic cells are unique professional APC that play critical roles immune responses [16]. Thus, we evaluated the effect of VSSP in DC expression of class II MHC and a variety of co-stimulatory molecules. Fig. 2 clearly shows that VSSP-treated DC (10 μg/ml) up-regulated CD80, CD86, CD40 and MHC II. Importantly, LPS (1 μg/ml) was indistinguishable from VSSP for inducing similar expression of these molecules.

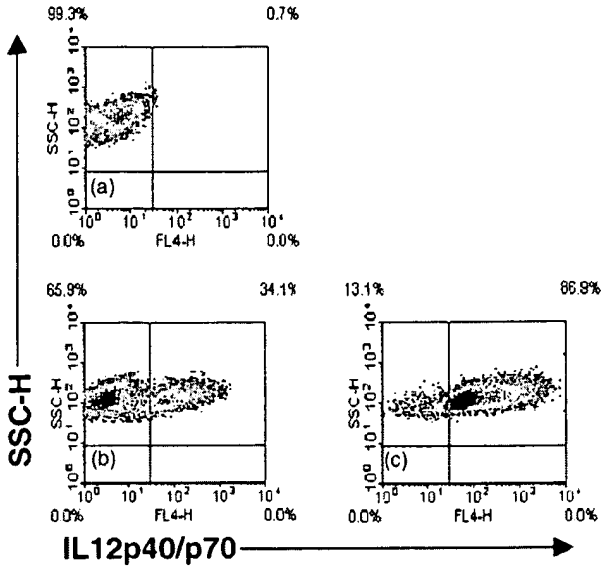

Fig. 3. IL-12p40/p70 production induced in bmDC by VSSP. Bone marrow-derived DC were stimulated with either medium (a), LPS (b) or VSSP (c) in the presence of Golgi Plug (Brefeldin A). Intracellular cytokine production was assessed after 24 h. Data are presented in density dot plots from a single experiment representative of four experiments yielding comparable results.

3.3. VSSP promote IL-12p40/p70 production

To evaluate the capacity of VSSP to induce IL-12 production, the intracellular cytokine levels in the gated dendritic cell population were measured by flow cytometry. Differences between LPS- and VSSP-treated DC were observed. Although a high percentage (87%) of IL-12p40/p70 producing DC were detected after stimulating with VSSP, only 34% of LPS stimulated DC produced this cytokine (Fig. 3).

3.4. VSSP induce activation of bmDC derived from LPS hyporesponsive mice

To investigate the possibility that components of VSSP other than LPS could induce DC maturation, we first determined the surface markers expression on LPS hyporesponsive C3H/HeJ mice-derived bmDC after culture with LPS (1 μg/ml), VSSP (10 μg/ml), medium alone or with an anti-mouse CD40 MAb (2 μg/ml) as a positive control [15]. As expected, the effect of LPS was weak as indicated by the low expression of MHC II, CD80, CD86 and CD40. Strikingly, VSSP induced the expression of these markers (Fig. 4).

We also tested if other components of VSSP, in addition to LPS, could promote DC activation. For this purpose, VSSP- and LPS-conditioned C3H/HeJ-derived bmDC cytokine and chemokine profiles were examined. Results from the RNA protection assay (Fig. 5) showed that both LPS and VSSP induced similar cytokines and chemokines pattern in

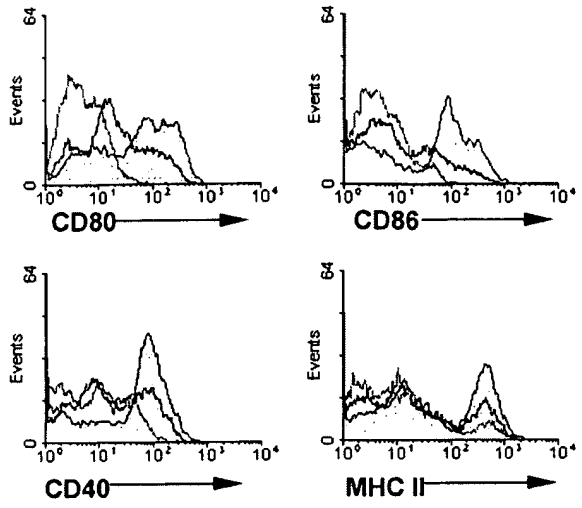

Fig. 4. Maturation of DC derived from the LPS hyporesponsive mice C3H/HeJ by VSSP. C3H/HeJ-derived CD11c$^+$ bmDC were cultured for 24 h with VSSP (light gray solid), LPS (black line), or medium (gray line) and expression of MHC II, CD40, CD80 and CD86 was examined by flow cytometry. Data are presented as histograms from a single experiment representative of three others.

bmDC from C57Bl/6 mice, while bmDC from C3H/HeJ remained hyporesponsive to LPS. In contrast, whilst only mRNA for IL-1α was absent, the rest of the induced genes in C57Bl/6 DC were also detectable on C3H/HeJ-derived DC in response to VSSP. In particular, IL-12p40, IL-6

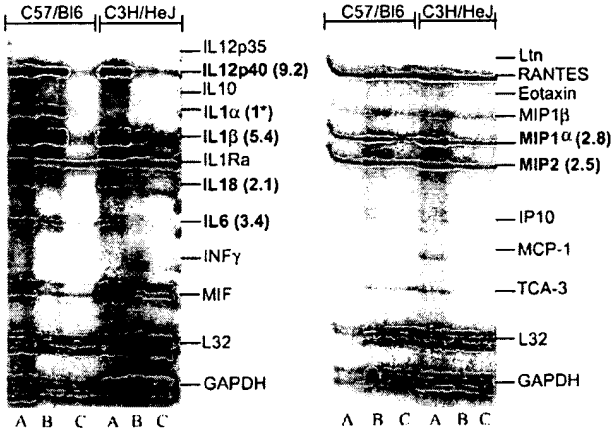

Fig. 5. Inflammatory chemokines and cytokines mRNA induced by VSSP in DC derived from normal and LPS hyporesponsive mice. CD11c$^+$ bmDC derived from C3H/HeJ or C57Bl/6 mice were incubated with VSSP (A), LPS (B) or medium (C) for 8 h and an RNAse protection assay was performed to determine induction of the indicated cytokines and chemokines at the mRNA level. In bold are those proteins for which the gene transcription was induced in C57Bl/6 DC ($DC^{AorB}/DC^{C} \geq 2$; after normalization). Numbers in brackets indicate the relative gene transcription induced by VSSP in C3H/HeJ DC given by the ratio of DC^{A}/DC^{C}, after normalization. For the rest of the evaluated cytokines and chemokines there was no induction of transcription ($DC^{A-B}/DC^{C} = 1$) in both mouse strains.

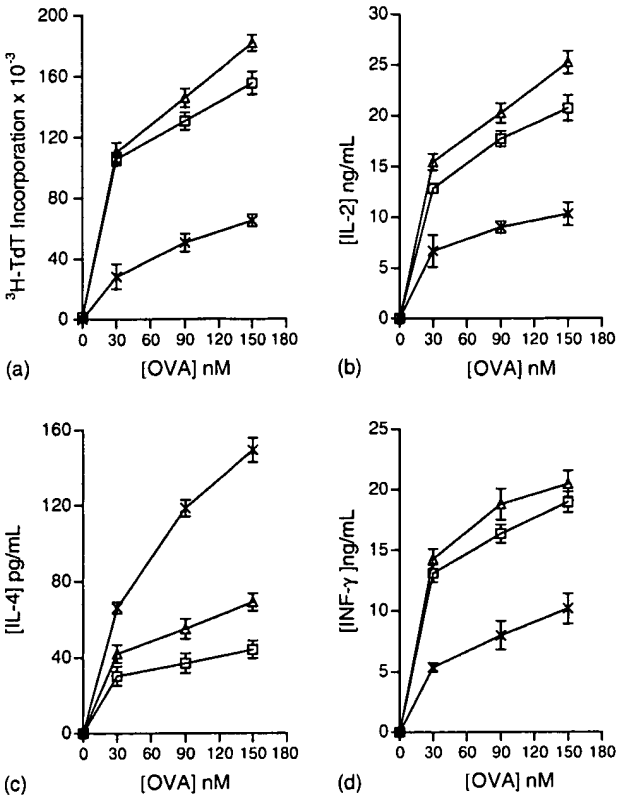

Fig. 6. Th1-associated OVA-specific responses promoted in naïve CD4$^+$ T cells by VSSP-treated DC. bmDC matured in the presence of VSSP (□), LPS (△) or media (×) were co-cultured with DO.11.10 Tg naive CD4$^+$ T cells at the indicated OVA concentrations. Figure shows: (a) DNA synthesis and (b) IL-2, (c) IL-4 or (d) INF-γ production. Data are presented as means ± S.D. from a single experiment representative of at least three other independent experiments.

and IL-1β were seen at high levels suggesting that VSSP contains components, in addition to LPS, that signal DC (Fig. 5).

3.5. VSSP promote an OVA-specific Th1 response on naïve T cells

Once we knew that VSSP can stimulate DC, the capacity of VSSP-treated DC to activate and drive antigen-specific Th1/Th2 responses on naïve T cells was studied. CD4$^+$ T cells purified from DO.11.10 mice were incubated with bmDC from each stimuli condition. VSSP- or LPS-matured bmDC revealed a DC phenotype that promoted increased proliferation of CD4$^+$/CD62^{high} Ag-specific Tg T cells at all OVA concentrations tested (Fig. 6a). To determine whether the observed increase in proliferation was associated with either Th1 or Th2 cytokine profiles, the secretion of IL-2, INFγ and IL-4 were evaluated. VSSP-treated bmDC promoted a Th1 profile of cytokine secretion in CD4$^+$ T cells (Fig 6b–d), characterized by an increased INFγ production and inhibited IL4 secretion.

Table 1
VSSP induces human DC maturation

Treatment	CD11c	CD83	CD86	CD40	HLA-DR
DC^0	36.7	2.0	2.7	2.6	35.0
DC^{LPS}	160.2	5.6	8.5	5.0	92.4
DC^{MPLA-A}	70.7	2.8	4.1	2.8	71.6
DC^{VSSP}	198.5	6.5	10.1	4.5	104.2

Data are representative of one of the three experiments expressed as MFI values calculated after the MFI values obtained with isotype control Mabs were subtracted.

3.6. VSSP is more effective than MPL-A in the activation of human DC

MPL-A is a derivative of LPS that retains most of its endotoxin bioactivities [17], but with attenuated toxicity [18,19]. It has been administered safely to humans in various clinical trials [20], so we compared the potency of VSSP relative to MPL-A on human DC activation. Monocyte-derived immature DC were cultured for 24 h in the presence of VSSP (1 μg/ml), LPS (0.1 μg/ml), MPL-A (1 μg/ml) or medium alone and the surface expression of several DC maturation markers was measured by FACS. DC treated with MPL-A (DC^{MPL-A}) had higher expression of CD11c, CD83, CD86 and HLA-DR than medium-treated DC (DC^0). However, the levels of these molecules, also including CD40, on DC^{VSSP} and DC^{LPS} were significantly greater than those of DC^{MPL-A} (Table 1).

4. Discussion

Previously, we have demonstrated that immunization with emulsified VSSP consistently induced IgM and IgG antibodies against highly tolerated gangliosides [10,11]. To define the advantage of using this new adjuvant in future vaccines OVA was mixed with VSSP emulsified in Montanide ISA 51 (IFA), VSSP alone or CFA (as a reference adjuvant) and compared the anti-OVA antibody titters elicited in the immunized mice. The same intensity in the antibody immune responses was observed using CFA and IFA/VSSP. A common feature of these adjuvants is that they are both formulated with bacterial products (*Mycobacterium tuberculosis* (Mt) and Nm for CFA and VSSP, respectively) and emulsified in incomplete Freund's adjuvant (IFA). This result suggests that VSSP could be a safer alternative of CFA that can be used in therapeutic vaccines for humans, at least for the induction of antibody responses. More interestingly, this experiment also shows that VSSP alone can be used as an adjuvant. The antibody titters induced in mice immunized with OVA/VSSP alone were significantly lower than those measured in the corresponding animals inoculated with OVA/emulsified VSSP. Nevertheless, the specific antibody levels induced in OVA/VSSP-treated animals were notoriously high (1.6×10^6). Moreover, VSSP has also shown its ability to stimulate OVA-specific T cell responses. Proliferation of spleen cells obtained from mice after one single dose of OVA formulated with CFA or OVA co-administered with VSSP were not significantly different. A number of bacterial products which are effective immunostimulants [2,3] have been reported. We demonstrated that VSSP belongs to this privileged group.

The fact that VSSP alone can stimulate the anti-OVA immune response means that we can probably eliminate the oily component-associated toxicity of emulsified vaccine formulations. Another very important advantage of VSSP is its own capability to exert an adjuvant effect without any covalent conjugation to the nominal antigen.

The discovery and characterization of adjuvants that promote a Th1 cell-mediated immunity is currently an important area in vaccine development. Our studies also addressed the ability of VSSP to modulate the differentiation of Th responses. Different subsets of DC act as a bridge between pathogens and T cells [21]. In this way, myeloid DC preferably induce Th1 differentiation while lymphoid DC induce Th2 [22]. However, a certain degree of flexibility on the ability of each DC subset in directing T cells responses has also been documented. This depends on signals from pathogens and the microenvironment [23]. Based on this "functional plasticity" of DC we have studied the roll of these cells on the Th1 response triggered by VSSP. Thus, firstly, we measured the capacity of VSSP to mature DC. Stimulation of bmDC for only 18 h with VSSP leads to increased expression of MHC II and co-stimulatory molecules compared with non-stimulated DC. This result was also obtained with human DC derived from monocytes. Interestingly, no differences on DC activation were found between VSSP and LPS. Due to its marked toxic side effects, LPS are not suitable for use in vaccines. On the other hand, certain adjuvants have been formulated using MPL-A, which has been reported to retain the immuno-stimulating activities of the parent LPS molecule [18], but has high attenuated toxicity [19]. Some of these adjuvants have been tested in animals models and clinical trials showing an acceptable profile of toxicity and efficacy [20]. Curiously, in this study, we also established that VSSP has a more powerful effect over human DC than MPL-A.

Results of the in vitro T helper cell assay, in which naïve CD4+ T cells from DO.11.10 Tg mouse were cultured with VSSP-matured bmDC in the presence of OVA, illustrated how VSSP preferentially stimulated T cells to secrete INFγ whilst IL-4 production was diminished. This model has been previously used to demonstrate opposite effects for LPS and a nematode-derived protein [24], validating the potent Th1 promoter effect of VSSP.

IL-12 produced by DC cells is pivotal for the development of Th1 responses [25]. The intracellular staining experiments showed a high level IL-12 production by DC in response to VSSP. IL-12 production by DC has been documented for

many gram-negative bacterial products like OmpA [2] and CpG [26]. Recently, the secretion of IL-12 by DC in response to *N. meningitidis* has been also reported evidencing that this phenomenon requires LPS expressed in the intact membrane of the bacteria [5]. However, the LPS requirement was questioned since the results from the RNase protection assay (RPA) showed that these nanoparticules are able to induce the transcription of the gene coding for the IL-12p40 (the inducible subunit of the active IL-12p70 [27]) in C3H/HeJ mice. In this experiment we also detected the induction of the IL-18 transcript on DC cultured with VSSP. IL-18 is a controversial cytokine regarding its link with Th1 or Th2 differentiation, but it is widely accepted that IL-18 acts in synergy with IL-12 to stimulate T cells to produce INFγ [28]. Another cytokine that came up with the RPA on VSSP-treated DC is IL-6. This constitutes an important finding since it has been recently reported that production of this cytokine by DC in response to TLR ligation during infection is critical for T cell activation. It renders antigen-specific T cells, refractory to the suppressive activity of $CD4^+CD25^+$ regulatory T cells [29].

In addition, to asses the role of LPS in VSSP, we studied the phenotype of VSSP-stimulated DC derived from the TLR4 (the transducing receptor for LPS [30]) mutant mice C3H/HeJ. As expected, DC maturation markers up-regulation, observed in normal mice after exposure to LPS, was abrogated in C3H/HeJ mice. Strikingly, VSSP was still able to induce normal levels of C3H/HeJ-DC maturation under the same conditions. In terms of DC cytokine and chemokine expressions in stimulated DC VSSP and LPS induced the same profile in normal mice while in C3H/HeJ mice a little up-regulation of some cytokines with LPS and a normal response with VSSP were observed. These data suggest that VSSP could additionally be engaging other receptors different from TLR4 to induce DC activation. The TLR4 involvement was evidenced by the lack of IL-1α up-regulation in C3H/HeJ DC after either VSSP or LPS exposure. These results may indicate that VSSP is composed of a variety of pathogen-associated molecular patterns (PAMP) capable of interacting with TLR4 and with at least one more pattern recognition receptor (PRR) on DC. This in addition to the complexity of VSSP, can be considered as an advantage because the repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between TLRs. It has been reported that when TLR2 and TLR6 are recruited together to the phagosome they interact with peptidoglycan and induce cytokine induction. By contrast, TLR2 recognizes another component without TLR6 [31].

In this paper, we propose that VSSP acts as danger signals that warns of *N. meningitidis* infection and activates immune defenses. These danger signals provided by VSSP may induce potent adjuvant function through DC activation and Th1 polarization. Therefore, VSSP could be a good alternative to the existing adjuvants for use in future vaccines, a concept that is currently under investigation.

Acknowledgements

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Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for dendritic cell activation

Circe Mesa[a,*], Joel de León[a], Kevin Rigley[b], Luis E. Fernández[a]

[a] *Vaccine's Department, Centre of Molecular Immunology, 216 esq 15, Atabey, Playa, CP 16040, C. Habana, Cuba*
[b] *Dendritic Cell Group, Edward Jenner Institute for Vaccine Research, Compton, Newbury, RG207NN, UK*

Abstract

Recent findings in the interactions between pathogens and the innate immune system, particularly with dendritic cells (DC), have opened new opportunities for adjuvants designs. We have elaborated a new approach, in which gangliosides are incorporated into the outer membrane complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP). VSSP demonstrated a unique ability to render highly tolerated gangliosides immunogenic. These results drove our attention to the immunopotentiating properties of VSSP. Here we examined the VSSP adjuvant effect on dendritic cell activation. Also the role of LPS on this effect was dissected. This study reveals that VSSP is a potent adjuvant for DC activation.

Keywords: Dendritic cells; *Neisseria meningitidis*; LPS

1. Introduction

Emerging theories and knowledge about the immune system regulation have strongly influenced in adjuvant development. We have described new cancer vaccines formulations based on OMV adjuvanticity properties and the hydrophobic incorporation of gangliosides to form very small size proteoliposomes (VSSP) [1]. Vaccination with $VSSP^{NAcGM3}$ increased the overall survival of mice challenged with the NAcGM3 positive melanoma B16 tumor [2]. This peculiar ability of VSSP to render immunogenic ubiquitous gangliosides, suggested strong immunopotentiatory properties for VSSP. In the present study, we established that the adjuvanticity of this formulation is mediated by a proper dendritic cells (DC) maturation.

2. Material and methods

2.1. Mouse bone marrow DC preparation

Bone marrow (BM) derived DC were prepared as described elsewhere. Once differentiated, BMDC were cultured with or without additional stimuli. DC was analyzed for expression of surface molecules by flow cytometry.

2.2. Human DC preparation

$CD14^+$ cells enriched from human PBMC were cultured in RPMI supplemented with 50 ng/mL of recombinant human (rh) GM-CSF and 1000 U/mL of rhIL-4. At day seven cells were cultured with or without additional stimuli and then analyzed for expression of surface molecules by flow cytometry.

3. Results

3.1. VSSP induce maturation of BMDC

We evaluated the effect of VSSP on DC and the possibility that components other than LPS could induce this effect. To do this, we first determined the surface markers expression on C57Bl/6 and LPS hyporesponsive C3H/HeJ mice-derived BMDC after culture with LPS (1 μg/mL), VSSP (10 μg/mL), medium alone. Table 1 clearly shows that VSSP up-regulated every molecule measured on both

* Corresponding author. Tel.: +53 7 2716810; fax: +53 7 2720644.
E-mail address: circe@ict.cim.sld.cu (C. Mesa).

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Table 1
VSSP induces DC maturation

DC	Treatment	CD11c	CD83	CD80	CD86	CD40	MHC II
Human	DC^0	36.7	2.0	n.d.	2.7	2.6	35.0
	DC^{LPS}	160.2	5.6	n.d.	8.5	5.0	92.4
	DC^{MPLA-A}	70.7	2.8	n.d.	4.1	2.8	71.6
	DC^{VSSP}	198.5	6.5	n.d.	10.1	4.5	104.2
C57Bl/6	DC^0	43.25	n.d.	21.88	10.66	7.7	60.66
	DC^{LPS}	86.48	n.d.	52.79	36.92	35.5	161.18
	DC^{VSSP}	83.88	n.d.	50.61	33.34	33.57	100.72
C3H/HeJ	DC^0	26.36	n.d.	6.95	7.31	15.73	17.77
	DC^{LPS}	33.45	n.d.	8.64	14.39	15.06	38.45
	DC^{VSSP}	80.59	n.d.	14.09	17.57	24.67	60.64

Data are representative of one of three experiments expressed as MFI values calculated after the MFI values obtained with isotype control. Mabs were subtracted. n.d.: not determined.

strains derived DC. Importantly, LPS was indistinguishable from VSSP on the C57Bl/6 for inducing similar expression of these molecules. However, on the C3H/HeJ obtained DC, the effect of LPS was weak compared with VSSP.

3.2. VSSP is more effective than MPL-A in the activation of human DC

We compared the potency of VSSP (1 μg/mL) relative to LPS (0.1 μg/mL) or MPL-A (1 μg/mL) on human DC activation. DC treated with MPL-A (DC^{MPL-A}) had higher expression of CD11c, CD83, CD86 and HLA-DR than medium treated DC (DC^0). However the levels of these molecules, including also CD40, on DC^{VSSP} and DC^{LPS} were significantly greater than those of DC^{MPL-A} (Table 1).

4. Discussion

These studies shows that both VSSP and LPS induce similar DC maturation. However, the adjuvanticity of VSSP can not be explained by the LPS component alone since VSSP induces DC activation in C3H/HeJ mice which are hyporesponsive to LPS. This suggests that, perhaps a pattern recognition molecule in addition to Toll 4 is required for VSSP signaling.

Again, VSSP has similar effects as LPS on DC. However, LPS has toxic side effects which make it not suitable for use in vaccines. On the other hand, MPL-A, which retain the immuno-stimulating activities of LPS, but has a greatly attenuated toxicity, resulted less powerful over human DC than VSSP. Thus, it is likely that VSSP are a save and effective way of utilizing the beneficial immunomodulation qualities of LPS whilst neutralizing its pyrogenic effect.

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Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant

Belinda Sánchez Ramírez[1]*, Eduardo Suárez Pestana[1], Greta Garrido Hidalgo[1], Tays Hernández García[1], Rolando Pérez Rodríguez[1], Axel Ullrich[2] and Luis Enrique Férnandez[1]

[1]*Vaccine Department, Center of Molecular Immunology, Havana, Cuba*
[2]*Department of Molecular Biology, Max-Planck-Institute for Biochemistry, Martinsried, Munich, Germany*

The epidermal growth factor receptor (EGFR) plays a central role in regulating neoplastic processes. The EGFR overexpression in many human epithelial tumors has been correlated with disease progression and bad prognosis. Passive EGFR-directed immunotherapy, but not active specific approaches, has already been introduced in medical oncology practice. Then we wonder if mice immunization with the extracellular domain of murine EGFR (mEGFR-ECD) in adjuvants can circumvent tolerance to self EGFR, by inducing an immune response with consequent antitumor effect. The present study demonstrated that despite mEGFR expression in thymus, strong DTH response was induced by inoculation of mice with the mEGFR-ECD. This self-immunization, using both CFA and very small sized proteoliposomes from *Neisseria meningitidis* (VSSP), promoted highly specific IgG titers, predominantly IgG2a and IgG2b. Sera from mice immunized with mEGFR-ECD/VSSP not only recognized EGFR+ tumor cell lines by FACS, but also inhibited their *in vitro* growth, even in the absence of complement. Noteworthy, vaccination of mice with mEGFR-ECD/VSSP stimulated a potent antimetastatic effect in the EGFR+ Lewis lung carcinoma model, while reproduction-associated side effects were absent. Curiously, mice immunized with the human EGFR-ECD (Her1-ECD) in VSSP though induced highly specific IgG antibodies with strong *in vitro* cytotoxic effect over EGFR+ human cell lines, showed low cross-reactivity with the mEGFR-ECD. These results further encouraged the development of the Her1-ECD/VSSP vaccine project for patients with EGFR+ tumors.

Key words: EGFR; self-immunization; cancer therapy

The epidermal growth factor receptor (EGFR) belongs to the erbB family of 4 closely related cell membrane receptors, also known as the Type I receptor tyrosine kinase family: EGFR or HER1/erbB1, first to be molecularly cloned,[1] HER2/erbB2, HER3/erbB3 and HER4/erbB4. The 4 receptors consist of an extracellular ligand-binding domain (ECD), a transmembrane domain and an intracellular domain with tyrosine kinase activity for signal transduction. EGFR plays a central role in regulating both development and neoplastic processes. Binding of their specific ligands, such as epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α) among others, induces receptor activation, modulation of cell proliferation and differentiation in normal tissues and tumors. Although expressed in nonmalignant cells, the EGFR can be found overexpressed or mutated in many human epithelial tumors such as breast,[2,3] lung,[4] prostate[5,6] head and neck,[7] colorectal,[8] pancreatic,[9] bladder,[10] vulva and ovarian tumors.[11] This overexpression has been correlated with disease progression and poor prognosis.[12,13] Activation of the EGFR signaling pathway in cancer cells has been shown to enhance cell proliferation, angiogenesis, tumor promotion and metastasis, and to decrease apoptosis. The potential of EGFR-targeted therapies for cancer treatment has increased the development of different passive agents. Passive immunotherapy with specific monoclonal antibodies (MAb)[14,15] and treatment with tyrosine kinase inhibitor drugs such as Iressa[16,17] and Tarceva[18] are currently undergoing clinical trials with promising results or are commercially available. On the other hand, active immunotherapy strategies to block the EGF from binding to its receptor are being clinically tested by vaccinating patients with EGF coupled to P64k recombinant protein from *Neisseria meningitidis*.[19]

In addition, EGFR-based active specific immunotherapy may be an alternative and complementary approach for the treatment of epithelial tumors, provided that induction of an immune response against self EGFR is feasible. Preclinical studies of both a DNA vaccine based on xenogenic EGFR-ECD and dendritic cells pulsed with self EGFR-ECD have been recently published, demonstrating the validity of this active immunotherapy.[20,21] Here an alternative, more simple, approach, based on vaccination with the mEGFR-ECD protein for exploring the possibility of circumventing tolerance to self EGFR, was proposed. We constructed DNA plasmids encoding the murine EGFR-ECD, which were stably transfected in mammalian cells, and the corresponding recombinant protein was used for vaccination protocols. Besides, we expressed the Her1-ECD to compare the relative efficacy of self and non-self-immunization and for evaluating the immune response specificity to EGFR+ human tumor cells. A strong DTH response and specific IgG titers with a TH1-associated subclass pattern were obtained by inoculation of mice with the self protein in very small sized proteoliposomes (VSSP) and complete Freund adjuvant. The corresponding immune sera showed *in vitro* antitumor effect, inhibiting EGFR+ tumor cells proliferation. mEGFR-ECD vaccination induced a potent antimetastatic effect in 3LL-D122 Lewis lung carcinoma. Antibodies obtained in mice immunized with Her1-ECD/VSSP evidenced a low cross-reaction with the parent mEGFR-ECD.

Material and methods

Construction of the expression vector encoding mEGFR-ECD and Her1-ECD

DNA encoding the extracellular domain of murine EGFR was amplified by PCR using total cDNA from mouse liver as template. The sense primer 5′-CGGAATTCCTCTCCCGGTCAGAGATGC-GAC-3′ includes EcoRI excision site, the initiation codon and 4 bp from EGFR signal sequence. The antisense primer 5′-CGGGAT-CCTCAAGATGGTATCTTTGGCCCAGATG-3′ is complementary to bp 1978–2000 in 3′ region and contains a stop codon (double underlined) and a BamHI excision site (single underlined). The PCR product, a 1.9-kb fragment, was cloned into EcoRI/BamHI sites of the pBluescript KS$^+$ vector. The fragment encoding for mEGFR-ECD was recovered using HindIII/BamHI enzymes and cloned into the pcDNA3 expression vector (Invitrogene, San Diego, CA), generating the mEGFR-ECD/pcDNA3 plasmid.

DNA encoding the extracellular domain of human EGFR (Her1-ECD) was amplified by PCR using the Her1Δ533/pRK5 plasmid as template. The sense primer 5′-GGGGTACCCTTCGGG-GAGCAGCGATGCGA-3′ includes a KpnI excision site (underlined), the initiation codon ATG and 3 bp from the signal sequence of Her1. The antisense primer 5′-GCTCTAGATCAGGACGGG-ATCTTAGGCCCA-3′ is complementary to bp 2103–2123 in the

***Correspondence to:** Center of Molecular Immunology (CIM), P.O. Box 16040, Havana 11600, Cuba. Fax: +53-7-272-0644, 53-7-273-3509. E-mail: belinda@cim.sld.cu
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3′ region, and contains a stop codon (double underlined) and an XbaI excision site (single underlined). The PCR product, a 1.9-kb fragment, was cloned into KpnI/XbaI sites of the pcDNA 3-expression vector, generating the Her1-ECD/pcDNA3 plasmid.

mEGFR-ECD and Her1-ECD sequences were confirmed, by dideoxy nucleotide sequencing analysis, to be identical with those previously reported.[22,23] All enzymes were supplied by Boehringer-Mannheim, Penzberg, Germany.

Cell lines

Ehrlich Ascites tumor (EAT, ECACC No. 87032503), 3LLD122, a metastatic variant of Lewis lung carcinoma,[24] the murine thymoma EL4 (ATCC TIB-39), human embryonic kidney (HEK293, ATCC CRL-1573), human epidermoid carcinoma A431 (ATCC CRL-1555) and human lung adenocarcinoma H125[25] cell lines were grown in DMEM (Gibco, NY, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone, Utah), 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin 100 U/ml and streptomycin 100 μg/ml (Life Technologies, Grand Island, NY). HEK293 transfectants were adapted to growth in HyQ PF 293 (protein-free medium from Hyclone, Utah).

Generation of HEK293 transfectants

HEK293 cells were grown in 6-well plates (1.75×10^5 cells/ml), and 8 hr later were transfected with 4 μg of mEGFR-ECD/pcDNA3 or Her1-ECD/pcDNA3 plasmids, using the calcium phosphate transfection system. Plates were incubated overnight at 3% CO_2, and then at 5% CO_2. Transfected cells were selected in medium containing 1,000 μg/ml of G418 (Geneticin, Sigma) starting 48 hr after transfection, for the generation of mEGFR-ECD/HEK293 and Her1-ECD/HEK293 stable cell lines. Mock transfection (with pcDNA3 vector) was used as a negative control.

Lectin- or antibody-mediated precipitations of the recombinant proteins

Supernatants from mEGFR-ECD/HEK293 or Her1-ECD/HEK293 cultures (2 ml) were mixed with 10 μl of lectin-agarose (a lectin from *Triticum vulgaris*, Sigma, St. Louis) or 1 μg of R3 MAb (a MAb specific for human EGFR extracellular domain; CIM, Havana, Cuba) plus 20 μl of Protein A-Sepharose (Amershan-Pharmacia Biotech, Uppsala, Sweden), respectively. Samples were gently shaken overnight at 4°C, and afterwards centrifuged for 1 min at 11,000*g*. The precipitated recombinant proteins were separated on SDS-PAGE 7.5% and visualized by silver staining.

Purification and immunoblotting of mEGFR-ECD and Her1-ECD

Recombinant proteins were purified from confluent cultures of the respective transfectants by affinity chromatography. EAH-Sepharose 4B (Amershan-Pharmacia Biotech) was covalently coupled to human recombinant EGF (hrEGF) (Center of Genetic Engineering and Biotechnology, CIGB, Cuba) or to R3 MAb for mEGFR-ECD and Her1-ECD purification, respectively. Equilibration and washing steps were performed with PBS/NaCl (1 M, pH 7.0) and protein elution with glycine (0.2 M, pH 2.8). Purity was assessed by densitometry, using a personal densitometer SI (Amershan-Pharmacia Biotech) and Imag Quant Software. Protein concentrations were assayed by Lowry's method.[26]

Purified proteins' identity was established by immunoblotting. Recombinant proteins (30 μg) were applied to 7.5% SDS-PAGE gels and transferred to PVDF membranes (Gelman, Ann Arbor, MI). After blocking with NEGT buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl (pH 7.5), 0.02% Tween 20, 0.04% gelatin), membranes were incubated with R3 (for Her1-ECD, data not shown) or 7A7 (for mEGFR-ECD) MAbs and proteins were visualized using horseradish peroxides-conjugated secondary antibodies followed by enhanced chemiluminescence (Perkin Elmer Life Sciences).

RT-PCR

Total RNA was isolated from Balb/c or C57BL/6 mice thymus using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The reverse transcription and polymerase chain reaction (RT-PCR) was performed using the SUPERSCRIPT™ one-step RT-PCR system. Used EGFR and β-actin primers were designed from the published sequences.[27] After PCR amplification, 10 μl of the RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide. Total RNAs from murine thymoma EL4 was used as negative control.

Mice and immunization protocols

Female C57BL/6 mice, aged 8–12 weeks, were purchased from the National Center for Laboratory Animals Production (CENPALAB, Havana, Cuba). All mice were kept under pathogen-free conditions. Animal experiments were approved by the Center of Molecular Immunology's Institutional Animal Care and Use Committee (CIM).

Mice ($n = 5$ or $n = 10$ for DTH and humoral response studies, respectively) were immunized with 50 μg of either mEGFR-ECD or Her1-ECD in FA adjuvant, complete for the first immunization and incomplete for the rest (Sigma) or in VSSP adjuvant, obtained from the combination of the outer membrane proteins of *Neisseria meningitidis* with GM3 ganglioside, in water/oil (Montanide ISA 51, Seppic, Paris, France) emulsion.[28] Immunizations were made, subcutaneously (sc) for FA-adjuvated or intramuscularly (im) for VSSP-adjuvated preparations, on days 0, 14 (for the DTH study) and 0, 14, 28 and 42 (for humoral response studies). Sera were extracted on days 0, 21, 35 and 56. Control groups received PBS/FA or PBS/VSSP.

DTH test

Seven days after the last immunization, mice were sensitized by intradermal injection with 50 μg of mEGFR-ECD in 50 μl of PBS in the right hind foot pad and by the same volume of PBS in the left foot pad. After 48 hr, mice foot swellings were measured using a plethysmometer (Ugo Basile, VA, Italy). Mice immunized with 100 μg of Keyhole Limphet Hemocyanin (KLH), (Sigma, Aldrich) in FA and sensitized with KLH in PBS were used as positive controls, while those injected with PBS/FA and sensitized with mEGFR-ECD were considered as negative controls. Differences in DTH between treatment groups were statistically validated by Kruskal Wallis and Dunn's multiple comparison test.

Enzyme immunoassay

Microtiter plates (High binding, Costar) were coated with 10 μg/ml of mEGFR-ECD or Her1-ECD in carbonate buffer (0.1 M, pH 9.6) and incubated overnight at 4°C. Plates were blocked with 5% calf serum in PBS/Tween-20, and sera dilutions in duplicate, from immunized mice ($n = 10$), or preimmune sera (as negative control) were incubated for 1 hr at 37°C. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma) was added and incubated for 1 hr at 37°C. After addition of *p*-nitrophenylphosphate (1 mg/ml) (Sigma), the optical density (OD) was measured at 405 nm using a microwell system reader (Organon Teknica, Salzburg, Austria). All washes were made with PBS/Tween-20. The Mann Whitney U test was used to assess statistical differences between individual time points in the humoral response kinetic. ELISA test background was 2 times the OD at 405 nm of preimmune sera, which coincides with the OD value for PBS.

For determination of serum IgG subclasses, secondary isotype-specific biotinylated rat anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies were used (PharMingen). Optimal secondary reagent dilutions were established by ELISA with 14F7 (IgG1-specific for NGcGM3), T3 (IgG2a-specific for CD3) and T4 (IgG2b-specific for CD4) MAbs (CIM), while R24 MAb (IgG3-specific for GD3) (kindly provided by Dr. Philip O. Livingston, Memorial Sloan Kettering Cancer Center, NY). Unpaired *t*-test was used to check statistically significant differences between sera dilutions.

ELISPOT

Specific inguinal lymph (LN) nodes spot forming cells (SFC) were obtained from mice immunized with Her1-ECD/VSSP and

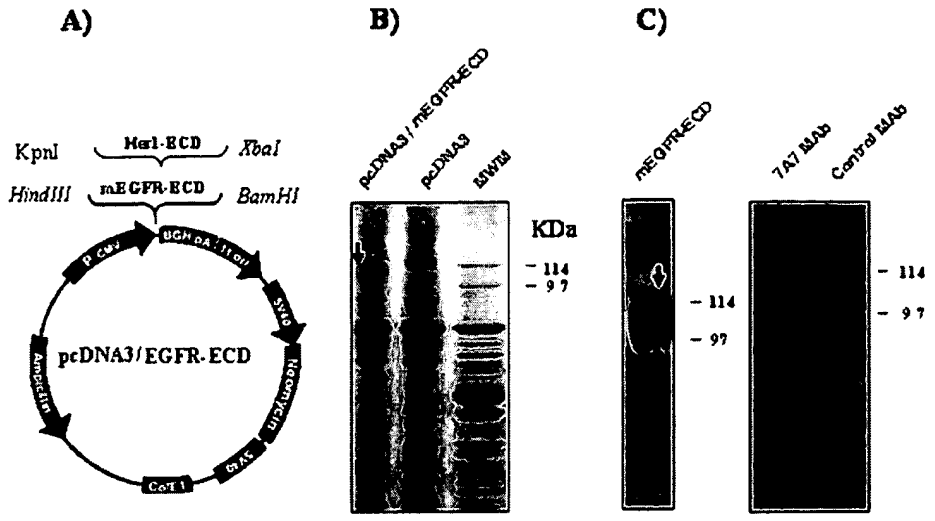

FIGURE 1 – Construction and functionality of the expression vector and purification of the mEGFR-ECD. (*a*) DNAs encoding the mEGFR-ECD or the Her1-ECD were inserted into the pcDNA3 expression vector. (*b*) mEGFR-ECD/pcDNA3 construct was verified by sequencing, and protein expression was checked by precipitation with lectin-agarose from supernatants of the HEK293 transfectant and visualized in 7.5% SDS-PAGE gels with silver staining. Mock transfection (pcDNA3) was used as control. (*c*) mEGFR-ECD purification was afforded by affinity chromatography with hrEGF-EAH Sepharose (left) and protein identity confirmation by Western blotting (right) using 7A7 MAb. An isotype control MAb was used as negative control.

tested by enzyme-linked immunospot (ELISPOT) as previously described,[29] with some modifications. Maxisorp 96-well plates (Nunc) were coated with 10 μg/ml of Her1-ECD or mEGFR-ECD in 50 μl carbonate buffer (pH 9.8) at 4°C overnight. After blocking with 5% BSA in PBS, different dilutions of pooled LN cells were incubated in triplicate for 6 hr at 37°C in a 5% CO_2 incubator. Antibodies secreted by individual cells were revealed as spots by the stepwise addition of 1.5 μg/ml of alkaline phosphatase-conjugated goat anti-mouse IgG (Fcγ) or IgM (Fcμ) antibodies (Jackson Immunoresearch laboratories, West Grove, PA) and the addition of 1 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma, San Louis, MO) in 0.1 M AMP buffer (pH 10.5) containing 0.6% agarose. Plates were incubated overnight at 4°C and the results were scored the next day by counting the number of specific SFC in a stereoscopic microscope. LN cells from mice injected with PBS/FA were used as negative control.

FACS analysis for EGFR recognition

Cells were stained with sera from immunized mice (1/200 dilution) followed by FITC-goat anti-mouse IgG (Jackson Immunoresearch laboratories). Up to 10,000 cells were acquired using a FACScan flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA). PCR and 7A7 MAb,[30] which is specific for mEGFR-ECD, were used to confirm EGFR expression in murine cells. Human EGFR expression in the corresponding cells was confirmed with R3 MAb. EL4 murine cell line and human lymphocytes were used as negative control cells.

Growth assay

Flat-bottomed 96-well microculture plates were seeded with 10^4 cells in 100 μl/well and grown in DMEM supplemented with 1% FCS in the presence of sera dilutions. After 48 hr of incubation at 5% of CO2, cells' viability was measured by the modified colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazodium bromide) assay.[31,32] Media were replaced by 100 μl/well of MTT (1 mg/ml) and plates were incubated under culture conditions for 4 hr. Formazan crystals were dissolved by addition of 100 μl/well of dimethyl sulfoxide followed by 30 min incubation at 37°C. Absorbance (OD) was measured at 540 nm using a microplate spectrophotometer and the reference wavelength (620 nm) OD subtracted. Background control contained only culture medium without cells. Cells without treatment were included as a maximum cell growth point. R3 and 7A7 MAbs were used as positive controls for human and murine cells lines, respectively. Statistical differences in the *in vitro* viability assay were evaluated by the unpaired *t*-test.

Cytotoxicity assay

Flat-bottomed 12-well microculture plates were seeded with 5×10^5 cells in 100 μl/well and grown in DMEM supplemented with 1% FCS in the presence of diluted sera (1/10). Sera from nonimmunized mice were used as control for unspecific complement-mediated cytotoxicity. To measure specific complement-independent cytotoxicity, immune sera were heated for 30 min at 56°C, and after 24 hr of incubation at 5% of CO_2, death cells were counted by FACS using propydium iodide. Cells without treatment were included as control for minimum death cell. R3 and 7A7 MAbs were used as positive controls for human and murine cells lines, respectively.

Tumor challenge assay

C57/BL6 mice (n = 10) were immunized, intramuscularly, 3 times biweekly with 100 μg of mEGFR-ECD/VSSP or PBS/VSSP. One day before the second immunization, mice were challenged with 2×10^5 tumor cells, subcutaneously, in the foot pad. Three weeks later, tumors reached 0.8 cm and were surgically removed. Twenty-one days after surgery, mice were sacrificed and spontaneous lung metastases quantified by weighing the lungs. Statistical differences between groups were determined by unpaired *t*-test.

Reproductive side effects studies

Female Balb/c mice (n = 10) were immunized with mEGFR-ECD/VSSP or PBS/VSSP (control group) as previously described. After checking the induction of specific antibodies against mEGFR-ECD, mice were mated with nonimmunized male animals. Fertility (number of mice completing pregnancy), number of pups, pups' birth weights and certain postnatal developmental features such as eyes opening, hair growth and incisor eruption were observed. The Mann Whitney U test was used to test statistically significant variations in the reproduction parameters between treated and control animals.

Statistical analyses

Variance homogeneity and data normal distribution were analyzed by Bartlett's and Bonferroni tests, respectively, using the

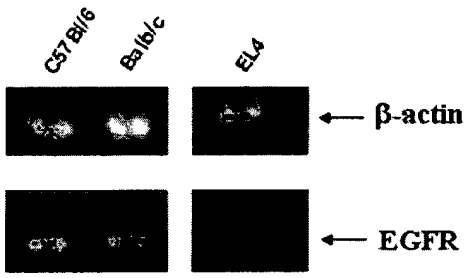

FIGURE 2 – EGFR is expressed in mice thymus tissue. Thymuses from C57/Bl6 and Balb/c mice were analyzed by RT-PCR for EGFR expression. Total RNA was extracted from thymuses of C57Bl/6 or Balb/c mice and from EL4 tumor (negative control) using TRIZOL reagent. RNA (1 μg) was reverse-transcribed to cDNA and the specific fragments amplified by PCR. β-actin mRNA served as an internal control.

SPSS version 10.0 software. All statistical tests (Kruskal Wallis and unpaired *t*-tests) were 2-sided and conducted using the Graph Pad Prism version 4.00 software. A probability value of $p < 0.05$ was considered as statistically significant.

Results

mEGFR-ECD and Her1-ECD expression and purification

cDNAs encoding mEGFR-ECD and Her1-ECD were successfully cloned into the pcDNA3 expression vector (Fig. 1*a*) and transfected in HEK293 cells as previous description. Expression of the soluble recombinant proteins by stable HEK293 transfectants was checked by lectin-agarose precipitation (mEGFR-ECD) or immune-precipitation with R3 MAb (Her1-ECD). In each case, as expected, a 105-kDa protein band was displayed but not for mock transfection, as determined by SDS-PAGE. Figure 1*b* shows the corresponding results for the mEGFR-ECD. mEGFR-ECD identity was further confirmed by affinity chromatography (with hrEGF-EAH Sepharose) purification combined with SDS-PAGE and western blotting, showing again the 105-kDa protein band (Fig. 1*c*). Achieved protein purity was 98% after only 1 purification step as determined by densitometry (data not shown).

mEGFR is expressed in thymus

As has been previously reported from studies with rat cell lines[33] and human thymus,[34] the EGFR presence in mice thymus was demonstrated by RT-PCR. The corresponding EGFR cDNA band (Fig. 2) became apparent when thymuses from C57BL/6 and Balb/c mice were analyzed, but not from murine thymoma EL4, used as a negative control.

mEGFR-ECD immunization induces DTH response

Generation of specific DTH responses was considered as a primary endpoint for the mEGFR-ECD vaccine (emulsified in FA) ability in immunizing mice. Mice were subcutaneously injected with mEGFR-ECD/FA and then sensitized with 50 μg of the nominal antigen. After 48 hr, mice foot swellings were measured, and inflammation scores in the vaccinated group animals were higher than those of the negative control group ($p < 0.05$, Dunn's multiple comparison test). Noteworthy, DTH responses induced in mice vaccinated with either mEGFR-ECD or KLH, a strongly immunogenic protein, were similar ($p > 0.05$) (Fig. 3).

mEGFR-ECD immunization induces a strong and long-lasting specific humoral response

Vaccine-related humoral responses were explored by immunizing C57BL/6 mice 4 times biweekly with 50 μg of mEGFR-ECD in 2 different adjuvants: FA, the reference adjuvant, and VSSP, a new product already clinically tested in humans. Inoculated mice developed high serum IgG antibody levels against the immunizing

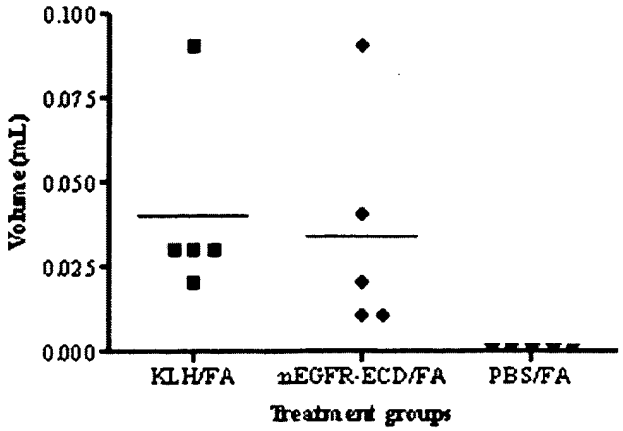

FIGURE 3 – mEGFR-ECD specific DTH response. Vaccine-induced DTH response was assayed by immunizing C57Bl/6 mice 2 times with 50 μg of mEGFR-ECD/FA. KLH/FA (50 μg) was used as positive control and PBS/FA as negative control. Mice included in the first and third groups were sensitized 7 days later with the mEGFR-ECD in PBS, while animals belonging to the second group were sensitized with KLH. mEGFR-ECD/FA-treated mice showed higher foot pad inflammations (Dunn's multiple comparison test, $p < 0.05$) than those of PBS control mice, but similar (Dunn's multiple comparison test, $p > 0.05$) to those in KLH group. A representative experiment from 2 independent ones is shown.

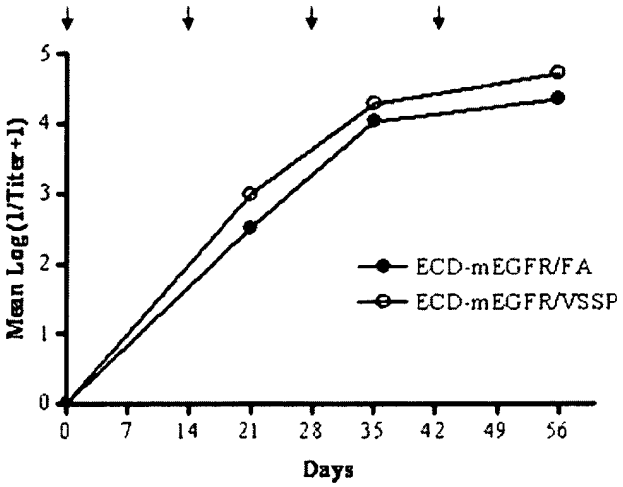

FIGURE 4 – Kinetics of the anti-mEGFR-ECD humoral response. Mice were immunized 4 times with 50 μg of the mEGFR-ECD in VSSP or FA biweekly. Antibody titers were quantified by ELISA in sera collected on days 0, 21, 35 and 56. Data was log transformed (1 + 1/titer) for graphic representation. While absent before the first vaccine administration (day 0), an increased antibody presence was detected after immunizations, preferentially when VSSP was used as adjuvant (Mann Whitney U test, $p < 0.05$). Immunization days are represented by arrows. A representative experiment from 3 independent ones is shown.

protein, which increased with successive immunizations, for both adjuvant formulations (Fig. 4). Indeed, the mEGFR-ECD/VSSP vaccine induced higher antibody titers than the FA one in each sera collection day (Mann Whitney U test, $p < 0.05$). Eight of 10 mice (80%) immunized with mEGFR-ECD/VSSP showed specific antibody titers above 1/40.000 by day 56, even reaching values up to 1/160,000, whilst only 2 of 10 mice (20%) rose titers above 1/40,000 in the mEGFR-ECD/FA immunized group (Table I). One year after having finished the immunization schedule and

TABLE I – RESPONSE FREQUENCY AND IgG TITERS[1] IN INDIVIDUAL ANIMALS BY DAY 56 AFTER IMMUNIZATION WITH THE mEGFR-ECD, USING FA OR VSSP AS ADJUVANTS

Treatment groups	Response frequency	1/IgG titer						
		2,500	5,000	10,000	20,000	40,000	80,000	160,000
mEGFR-ECD in FA	10/10	1	1	3	3	1	1	
mEGFR-ECD in VSSP	10/10		1		1	2	4	2

[1]Assayed by ELISA.

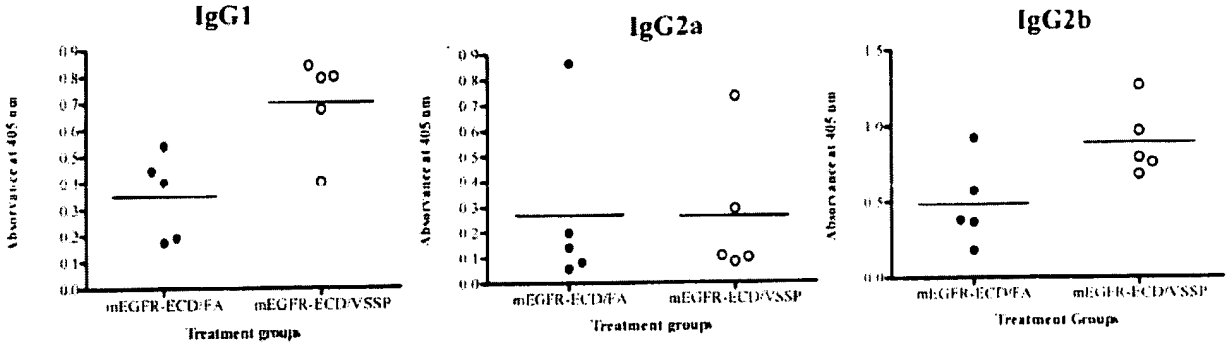

FIGURE 5 – IgG subclasses induced by immunization. Serum IgG subclasses pattern in C57Bl/6 mice after 2 inoculations with 50 μg of the mEGFR-ECD in VSSP or CFA biweekly, was measured by ELISA with samples collected on day 21, and diluted 1/10,000. Each point represents the mean absorbance value of duplicate samples in individual mice ($n = 5$). IgG2b levels were higher (unpaired t-test, $p < 0.05$) in the group immunized with mEGFR-ECD/VSSP, while no differences were found for IgG2a. A representative experiment from 3 independent ones is shown.

without antigen recall, the sera-specific IgG levels decreased in both groups of mice, although an appreciable response was still detected in 100% of animals (1/1,000 and 1/100 sera dilutions) (data not shown).

Administration of mEGFR-ECD in FA or VSSP to Balb/c mice produced elevated IgG titers in 100% of animals, similarly to what was observed in the C57Bl/6 case (data not shown).

Vaccination with mEGFR-ECD polarizes systemic immunity to a TH1 pattern

Consistent with previous results demonstrating that complete FA preferentially promotes a TH1 type response to the accompanying antigen,[35] elevated levels of IgG2a, IgG2b, and IgG1 were detected in day 21 sera corresponding to mice immunized with mEGFR-ECD/FA. As shown in Figure 5, while no differences in IgG2a levels were found in sera of mice vaccinated with FA or VSSP formulations (unpaired t test, $p > 0.05$), the use of VSSP promoted the induction of higher levels of IgG2b ($p < 0.05$).

Her1-ECD/VSSP immunization generates specific B cell clones with low cross-reactivity to the mEGFR-ECD

We wonder if immunization with the xenogenic EGFR-ECD could induce antibodies reacting with the self EGFR. C57BL/6 mice were immunized with 50 μg of Her1-ECD in FA or VSSP. All immunized mice, independent of the used adjuvant, developed high IgG antibody titers against the human protein (1/320,000) by day 56 (data not shown). Alternatively, ELISA experiments reflected an evident but low cross-reactivity with the murine protein by day 21 (Fig. 6*a*). This low cross-reactivity against the mEGFR-ECD was confirmed by ELISPOT assay. Sixty-two IgG and 6 IgM secreting specific SFC in 10^6 LN cells were found in LN of Her1-ECD-immunized mice, while only 21 IgG secreting SFC/10^6 LN cells cross-reacted with the mEGFR-ECD (Fig. 6*b*).

Immune sera recognizes full length EGFR by FACS

To check whether immunizations with a truncated EGFR affected the recognition of the full length EGFR in its native conformation on the cell surface, EGFR+ cells were analyzed by FACS. EAT[36] and 3LL-D122 murine cell lines were positively stained by sera from mice immunized with mEGFR-ECD/VSSP (Fig. 7*a*). Besides, sera from mice immunized with Her1-ECD/VSSP readily reacted with A431 and H125 cell lines (Fig. 7*b*). Sera from control mice, immunized with PBS/VSSP, neither recognized murine nor human EGFR+ cells.

Immune sera inhibit EGFR+ tumor cells growth and possess cytotoxic effect

To determine whether immunization with mEGFR-ECD/VSSP or Her1-ECD/VSSP can generate serum antibodies affecting murine or human tumor cells growth *in vitro*, the MTT viability assay was performed. Incubation of 3LL-D122 or H125 cells with sera obtained from mice immunized with mEGFR-ECD/VSSP or Her1-ECD/VSSP, respectively, decreased the number of viable cells if compared with preimmune sera after 48 hr, and this effect was sera-dilution-dependent (unpaired t-test, $p < 0.05$) (Fig. 8). In addition, the immune sera *in vitro* cytotoxicity over EGFR+ cells was evaluated after treating cells for 24 hr with the corresponding complement inactivated samples, following propydium iodide staining. FACS analysis showed that treatment of 3LL-D122 cells with inactivated sera produced 55.83% of death cells, suggesting that a complement-independent cytotoxicity mechanism is operating (Fig. 9*a*). In parallel, H125 cells were treated with sera from mice immunized with Her1-ECD/VSSP, and the effects over the cells evaluated. Incubation with immune sera manifested cytotoxic effects (FACS) (Fig. 9*b*).

Antimetastatic effect of mEGFR-ECD/VSSP vaccination

To investigate whether the autologous vaccination can protect individuals from metastatic widespread, mice were immunized with the mEGFR-ECD in VSSP and, 1 day before the second immunization, challenged with 2×10^5 tumor cells in the foot pad. Three weeks after malignant cells inoculation, tumors were surgically removed. Twenty-one days after surgery, mice were sacrificed and the spontaneous lung metastases quantified by weighing the lungs. As shown in Figure 10, vaccination of mice significantly reduced lung metastasis ($p < 0.01$), compared with animals in the control group.

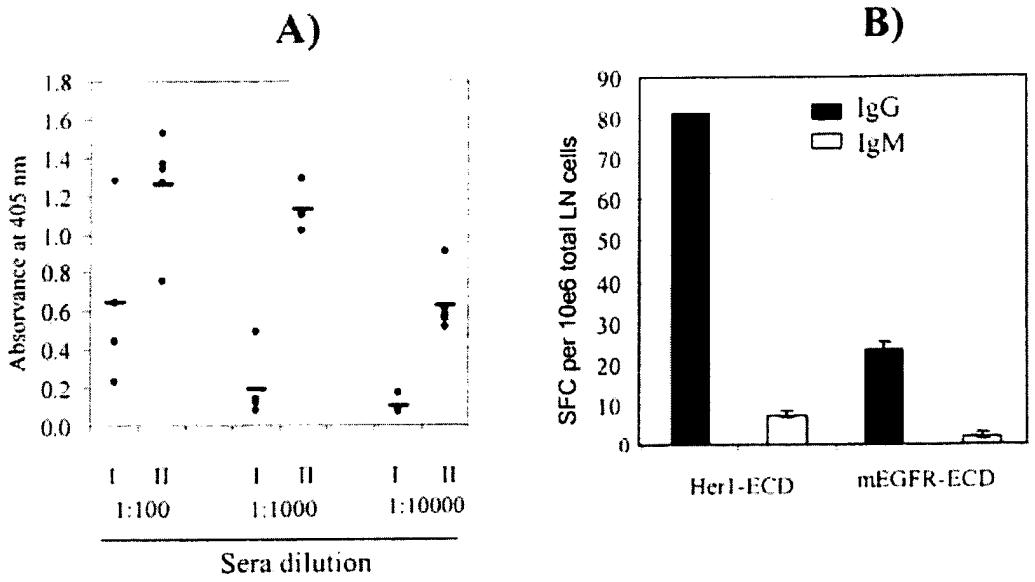

FIGURE 6 – Immunization with Her1-ECD/VSSP mobilizes B cell responses. Mice were immunized 2 times with 50 μg of Her1-ECD/VSSP biweekly. (*a*) Specific IgG antibodies against the Her1-ECD (II) and their cross-reaction with mEGFR-ECD (I) were assayed by ELISA employing sera collected on day 21. (*b*) Specific SFC, secreting IgG and IgM antibodies against the Her1-ECD and their cross-reaction with the mEGFR-ECD were measured by ELISPOT. A representative experiment from 2 independent ones is shown.

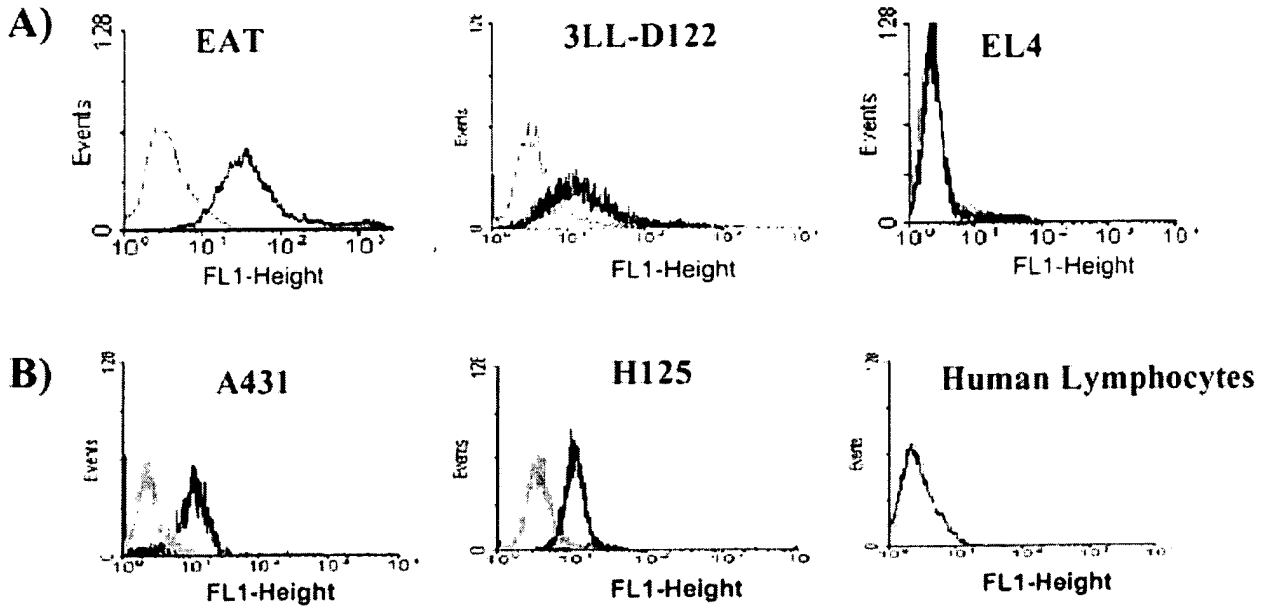

FIGURE 7 – Immune sera recognize EGFR+ tumor cells. (*a*) 1/200 sera dilutions, obtained from mice immunized with mEGFR-ECD/VSSP, reacted with 3LL-122 and EAT cell lines (black line) but not with EL4 cells. (*b*) 1/200 sera dilutions, obtained from mice immunized with Her1-ECD/VSSP, reacted with A431 and H125 cell lines (black line) but not with human lymphocytes. Sera from mice immunized with PBS/VSSP (gray line) were used as negative control.

Absence of reproductive side effects in mice immunized with mEGFR-ECD/VSSP

The potential side effects of "self" immunization in humans were stressed by examining the appearance of possible toxic symptoms in female mice immunized with the mEGFR-ECD in VSSP. After the vaccine, inoculation animals were mated and their progeny studied. Pregnancies rates were 5 out of 10 in the immunized group, while 3 out of 10 in control mice. The median number of pups per litter in both groups was 6 (range 5–7). Features like newborns' weights, hair growth, eyes opening and incisor appearance did not comparatively differ (Mann Whitney U test, $p > 0.05$) (Table II). On the other hand, a group of mice were observed for 1 year after fulfilling the immunization protocol, and the vitality, temperature and food intake were completely normal, without changes in functional hepatic parameters when compared with those of nonimmunized mice (data not shown).

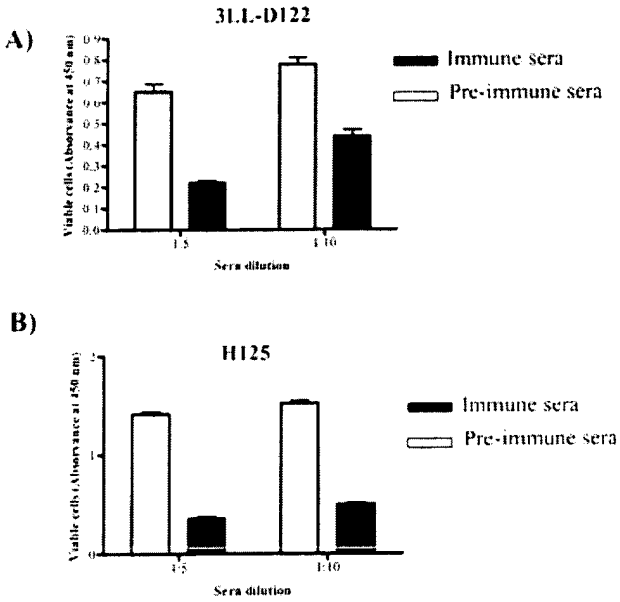

FIGURE 8 – Immune sera inhibit EGFR+ tumor cells growth. (*a*) 3LL-D122 cells or (*b*) H125 cells were grown in the presence of sera obtained from mice immunized with the mEGFR-ECD or the Her1-ECD, respectively. Preimmune sera were used as negative controls. After 48 hr, cells' viability was measured by the MTT colorimetric assay. Both kinds of hyperimmune sera were able to significantly decrease viable cells number (unpaired *t*-test, $p < 0.05$). Each bar represents the mean absorbance ± SD of 2 independent experiments.

Discussion

Despite the EGFR wide expression in the organism, it can be considered as a tumor-associated antigen (TAA) because of its overexpression in many epithelial tumors,[37] its implication in tumor growth and correlation with bad prognosis.[12] For that reason, the EGFR has become an attractive target for cancer therapy, and many attempts are currently ongoing, using this molecule as target for passive therapy with tyrosine kinase inhibitors and MAb.[38–40] Our results demonstrated that the murine EGFR extracellular domain, in an appropriate adjuvant, is enough immunogenic in mice to promote a strong antimetastatic effect in a relevant EGFR+ tumor model, highlighting this particular approach as an attractive new strategy for cancer treatment.

TAA tolerance represents a significant challenge for effective immunotherapy of human cancer, and in any successful vaccine strategy, this issue should be conveniently addressed in advance. The experimental demonstration that breaking self tolerance is possible has been recently published and is generally accepted under the principle that self recognition is a physiological phenomenon.[41] The fact that the EGFR is widely distributed in normal epithelial tissues hardly suggests that this molecule could be a very poor immunogen, introducing additional difficulties to any vaccine design. In our vaccine approach, the construction of chimerical molecules was avoided, introducing instead potent TH1 adjuvants. Emulsifying the recombinant protein either in FA or VSSP (a product already clinically tested in humans) renders a rather simple formulation. As the primary endpoint for the vaccine induced mEGFR-ECD immunogenicity, the induction of DTH was considered. Surprisingly, mEGFR-ECD/FA vaccination promoted severe inflammations in mice foot pads after the specific sensitization, similar to those mice previously immunized with KLH/FA and sensitized with this "foreign" protein.

Immunization of mice with the mEGFR-ECD in FA or VSSP also stimulated the specific humoral immunity, characterized by elevated IgG antibody titers, successively incremented with reimmunizations, an indicative of a mature response. Both adjuvants influenced IgG subclasses distribution in favor of IgG2a and IgG2b, an indirect indication of TH1 differentiation. Particularly, higher levels of IgG2b were associated with VSSP formulations. An indicative that the immune reaction against this kind of self proteins is limited came from other member of the EGFR family, Her2. Monkeys were successfully immunized only after 6 immunizations with the Her2-ECD, formulated in the powerful adjuvant Detox, and specific IgG titers never reached 1/10,000,[42] a value lower than that induced in mice with the mEGFR-ECD in FA or VSSP. Besides, Disis *et al.*[43] have shown that a Her2 neu-peptide-based vaccine, but not a whole-protein vaccine, can elicit humoral and cellular responses in rats. The EGFR is a tolerated self-antigen for which a specific T cells thymic deletion mechanism could be operating, requiring the presence of the auto-antigen in the thymus.[44,45] The EGFR thymic expression, although earlier reported in humans[34] and rats,[33] was confirmed in our lab for Balb/c and C57Bl/6 mice by RT-PCR. Similar to what has been reported in normal people, a total absence of anti-EGFR natural auto-antibodies was also observed in mice sera by ELISA and FACS. Nevertheless, evidences coming from cancer patients indicated that the presence of discrete serum anti-EGFR natural antibodies can be detected with certain frequency.[46] The same observations have been reported for breast cancer patients in which anti-Her2 antibodies and CTL could be measured,[47] lacking enough efficacy in preventing tumor progression. This "natural" immunity to Her2, present only in a minority of patients overexpressing the receptor, is of low magnitude.[48] The low, natural immune responses to Her1 and Her2 in cancer patients means that any associated target-directed therapeutic vaccine must efficaciously stimulate naïve B and T lymphocytes. In fact, Her2 vaccines, constructed with synthetic peptides mixed with granulocyte-macrophage colony stimulating factor as adjuvant, have been clinically tested in breast, ovarian and lung cancer patients overexpressing Her2 and 68% of them developed T-cell immunity against the self Her2 protein.[49]

More likely explanations for the unusually strong immunogenicity of the autologous Her1 protein observed in this study might be the full length EGFR truncation, the adjuvant conditioning of the antigen presentation context or both. EGFR truncation could modify the T cells repertoire immunodominance, favoring the presentation of cryptic determinants.[50] In this case, protein truncation did not affect the full length EGFR recognition in its natural conformation in the cells by the vaccine-induced serum antibodies, as determined in FACS experiments. This result suggests certain differences with the Her2 model, in which specific antibodies were undetectable in sera obtained from rats immunized with the rat Her2 intracellular domain (ICD), while CTL and antibodies with degenerated specificities for the human and rat Her2/neu were produced when the inoculated immunogen was the highly homologous foreign human ICD.[51]

The use of potent TH1-type adjuvants in combination with poorly immunogenic self proteins to promote a proinflammatory context for loosing the regulatory cells circuit was an attractive, tested idea. In this sense and as usual in experimental vaccine approaches complete FA was selected as reference TH1 adjuvant.[35] While complete FA cannot be used in human vaccines, a new, already clinically tested adjuvant (VSSP) with peculiar immune-modulatory properties was introduced in the EGFR-ECD vaccines. VSSP monotherapy in mice induced elevated IgG levels with a TH1-related pattern against GM3, a poorly immunogenic ganglioside,[52] and dendritic cell maturation with IL-12 production,[52] which in turn is pivotal for proinflammatory responses.[53,54]

Although the amino acid sequence homology between human and murine EGFR-ECDs is about 87%, sera obtained from mice immunized with the Her1-ECD formulation were unable to appropriately react with the murine protein, while only 1/3 of the vaccine-stimulated B cells, secreting specific IgG antibodies, cross-reacted with the mEGFR-ECD. The finding that serum-specific antibodies, induced in mice by immunization with the corresponding EGFR-ECDs in VSSP, caused the slow growing of EGFR+

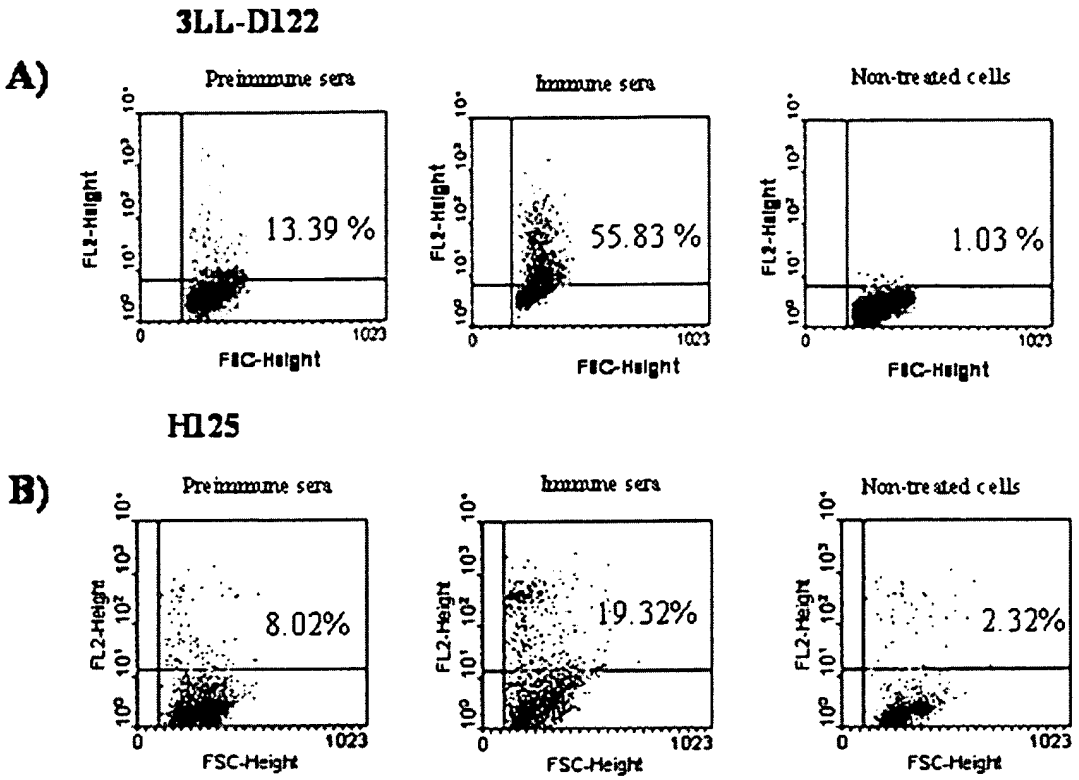

FIGURE 9 – Immune sera cytotoxic effect over EGFR+ cells. (*a*) 3LL-D122 and (*b*) H125 cells were incubated for 24 hr with inactivated complement sera from mice immunized with mEGFR-ECD/VSSP or Her1-ECD/VSSP, respectively. The immune sera cytotoxic effect was determined by FACS analysis. 7A7 (50% of cytotoxicity) and R3 (25% of cytotoxicity) MAbs were used as positive controls for 3LL-D122 and H125 cells, respectively. This experiment is representative of 2 independent ones.

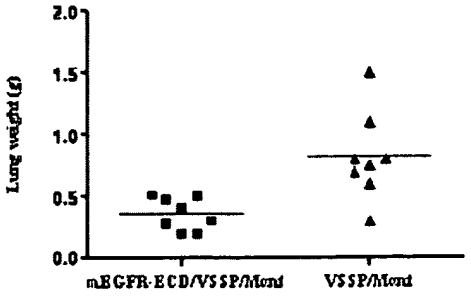

FIGURE 10 – Antimetastatic effect of mEGFR-ECD/VSSP vaccination. Mice were immunized 3 times with 100 μg of mEGFR-ECD/VSSP biweekly. One day before the second immunization, mice were challenged with 2×10^5 3LL-D122 tumor cells in the foot pad. Three weeks after malignant cells inoculation, tumors were surgically removed, and 21 days later, mice were sacrificed and the spontaneous lung metastases quantified by weighing the lungs. In vaccinated animals lung weights were significantly reduced ($p < 0.01$, unpaired *t*-test) compared with that of the corresponding control group. This experiment is representative of 2 independent ones.

tumor cells and also promoted a strong target-directed complement-independent cytotoxic effect emphasize the quality of the vaccine-induced immune response.

Further results associated with the *in vivo* mEGFR-ECD/VSSP antimetastatic effect in the EGFR+ 3LL-D122 Lewis lung carcinoma model are encouraging if the intermediate character of the selected experimental setting (rather therapeutic than prophylactic) was considered. Even though in this case tumor cells were inoculated into mice foot pads just after vaccine priming, supplying afterwards the 2 booster injections, a significant decrease in lung metastases number, after primary tumor surgical removal, was evident.

One week after the third vaccine administration, and coincident with the surgical removal of primary tumors, specific IgG titers in mice sera were elevated as noticed from the humoral response kinetics, probably suggesting that the induced antibodies might have a role in avoiding tumor cells' dissemination or in turn the growth of malignant cells already lodged in the lungs, or both. Future studies stressing the relative contribution of humoral and cellular immunity in the vaccine-induced antimetastatic effect, through hyperimmune serum transfer and the appropriate lymphocyte subsets depletion experiments are currently ongoing. The "spontaneous metastasis" 3LL Lewis lung carcinoma model, employed in this study, is rather significant because of the most likely resemblance to the real clinical situation in which surgeons frequently remove patients' primary tumors successfully, but unfortunately and more commonly, disease spreading will follow. Indeed, avoiding distant metastatic dissemination could be the most appropriate task for effective cancer vaccines. Interestingly, other 2 active immunotherapy approaches targeting the EGFR have shown recently *in vivo* efficacy in the Lewis lung carcinoma model but in a primary tumor scenario. A DNA vaccine,[20] based on the xenogenic EGFR-ECD gene and a self EGFR-ECD protein pulsed dendritic cells vaccine[21] were able to keep alive 60% of mice preventatively injected and afterwards challenged with LL/2c tumor. Although together all these results strongly suggest that EGFR could be a significant target not only for passive but also for active immunotherapy, another crucial remaining question is the better vaccine approach to follow up in the upcoming future clinical trials. A relevant learning from the present work is that

TABLE II – PREGNANCY AND NEWBORN REPRODUCTIVE PARAMETERS MEASURED AFTER mEGFR-ECD/VSSP IMMUNIZATION

Group	Fertility	Number of pups per litter	Weight of 1-day-old pups (g) (mean ± SD)	Hair growth (d.a.b.)[1]	Eyes opening (d.a.b.)	Incisor eruption (d.a.b.)
Treated	5/10	5–7	1.298 ± 0.03	5–7	13–15	10–13
Control	3/10	5–7	1.33 ± 0.07	5–7	13–16	10–13

[1]d.a.b. = days after birth.

just the use of the recombinant self protein in a potent adjuvant, like VSSP, could be appropriate for establishing an antitumor immunity in patients with EGFR+ tumors, indicating the existence of a new opportunity in this particular target, different from sophisticated vaccine technologies like the dendritic cells approach, or up to now ineffective vaccine formulations in humans like naked DNA.

EGFR-targeted therapies are expected to produce side effects related to the induction of autoimmunity. In fact, some side effects as skin rash have been reported for some related drugs,[55] but important toxic effects have not been found for the majority of the different approaches tested in clinical trials. As an example THERACIM, an anti-EGFR MAb (humanized R3, CIM), has been clinically tested (Phase II trials), in combination with radiotherapy, in head and neck cancer patients, raising up to 600 mg/cycle, without the detection of skin rash symptoms. Although it has been reported that EGFR gene expression inhibition is critical for cancerous cell growth but not for normal cells,[56] active immunization, providing a long lasting specific immune response, should be carefully monitored for possible side effects. The conducted immunization experiments with mEGFR-ECD/VSSP showed that while 1 year after the last vaccine booster anti-mEGFR-ECD IgG low levels were detectable in most animals, signs of toxicity were absent and functional hepatic parameters behave as in naïve mice.

Induction of EGF deficiency in rats affects the development of fetal but not adult tissues.[57] Considering the role of EGF in the epigenetic regulation of fetal and neonatal development, we studied the effect of anti-EGFR immunity in female mice fertility and their progeny. Marked side effects in these animals as a consequence of vaccination were not detected.

We conclude that self EGFR-ECD is rather immunogenic in FA or VSSP contexts. Taken together, the immunization approach described here may be an attractive and novel strategy for EGFR+ cancer active immunotherapy.

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Immunotherapy with CTL peptide and VSSP eradicated established human papillomavirus (HPV) type 16 E7-expressing tumors

Isis Torréns [a,*], Osmany Mendoza [a], Aileen Batte [a], Osvaldo Reyes [a], Luis E. Fernández [b], Circe Mesa [b], Gerardo Guillén [a]

[a] *Department of Cancer, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*
[b] *Department of Vaccines, Center of Molecular Immunology, 216 esq 15 Atabey, CP 16040, Havana, Cuba*

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Abstract

Peptide-based vaccines aimed at the induction of effective T-cell responses against established tumors have not been successful in clinic and require the use of new adjuvants. One of those is a new adjuvant in which gangliosides are incorporated into the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP). In a preclinical model of human papillomavirus HPV16-induced cervical cancer we show that vaccination with HPV 16 E7 derived minimal CTL peptide and VSSP protects mice against tumor challenge, induces regression of established tumors and produces E7-specific $CD8^+$ T-cell responses.

Keywords: Immunotherapy; HPV; VSSP

1. Introduction

Cervical cancer is the second cause of women cancer mortality in the world [1]. Human papillomaviruses (HPV), particularly HPV-16, are associated with most cervical cancers [2]. More than 99% of cervical cancers and their precursor's lesions, squamous intraepithelial lesions (SILs), contain HPV DNA [3]. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are co-expressed in most HPV-containing cervical cancers [4]. Therefore, these oncogenic proteins represent ideal target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated tumors [5].

In the past, most HPV research focused on E7, and therefore an E7 immunodominant epitope. Vaccination with a synthetic MHC class-I H-$2D^b$-binding peptide, HPV16 $E7_{49-57}$ (E7(p)), mixed with incomplete Freund's adjuvant (IFA) prevented tumor formation by HPV16-transformed B6 mouse embryo cells and elicited CTL responses in C57BL/6 mice [6].

Over the years, various vaccines containing exactly fitting MHC class-I binding peptides have been tested for their therapeutic efficacy in patients with progressive disease associated to HPV [7–9]. Although moderate T-cell responses were observed, the clinical benefit was only modest. This may be due to failure of the vaccines to induce strong sustained immunity because of impaired immune system, which is commonly found in patients diagnosed with cancer.

Recently, it has been demonstrated that $CD4^+$ T helper (Th) cells contribute to the development and efficacy of antitumor responses [10,11], probably due to their capacity to deliver essential activation signals to professional antigen presenting cells, such as dendritic cells (DC), needed for an optimal priming of tumor specific CTL [12–14]. Several studies show that effective CTL priming can be induced by: (a) the inclusion of Th epitopes in peptide vaccines [15,16]; (b) the use of long (32- to 35-aa) overlapping peptides including Th and CTL epitopes [17]; (c) the use of molecularly defined strong DC-activating adjuvants, such as monophosphoryl lipid A (MPL) [18,19], oligodeoxynucleotide (ODN)-CpG

* Corresponding author. Tel.: +53 7 2716022; fax: +53 7 336008/2714764.
E-mail address: isis.torrens@cigb.edu.cu (I. Torréns).

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[20–22], anti-CD40 Ab [23], GM-CSF [24] and ISCOM [25].

A new adjuvant has been previously developed in which gangliosides are incorporated into the outer membrane protein complex (OMPC) of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP) [26]. A recent study reveals that VSSP have the ability to activate mouse and human DC, in vitro and in vivo, with the corresponding IL-12p40/p70, TNF-α and IL-6 production [27]. Therefore, VSSP is a potent adjuvant for DC activation and Th1 differentiation.

In the present study, we demonstrate that immunization of C57BL/6 mice with a vaccine based in adjuvancity of E7(p) + VSSP without oily component protects animals against challenge with the E7-expressing murine tumor cell line, TC-1 [28], and also protects them against re-challenge with higher doses of TC-1 cells. Similarly, immunization of tumor-bearing mice with E7(p) + VSSP leads to tumor regression and long-term survival and produces E7-specific $CD8^+$ T-cell responses.

This is the first study to demonstrate that therapeutic immunization with a minimal CTL peptide and VSSP induces tumor regression and represents the first example of cancer immunotherapy based on the use of VSSP with a viral antigen.

2. Materials and methods

2.1. Mice

C57BL/6 mice (6–8 weeks old) were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). All animals were maintained under specific-pathogen-free conditions at the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). The experimental methods were approved by the Animal Care and Use Committee of the CIGB and were conducted in accordance with the Health Guide for the Care and Use of Laboratory Animals (HGCULA).

2.2. Cells

TC-1 cells were kindly provided by T.C. Wu (Johns Hopkins University, Baltimore). They were generated by co-transformations of primary C57BL/6 mouse lung epithelial cells with HPV-16 E6 and E7, and an activated *ras* oncogene as previously described [28]. The EL4 tumor cell line was obtained from American Culture Collection (TIB-39).

2.3. Peptides

All peptides were synthesized on solid phase and purified by reverse-phase high-performance liquid chromatography to >95% purity on an acetonitrile/H20-trifluoroacetic acid gradient and confirmed by ion-spray mass spectrometry (Micromass, Manchester, United Kingdom). Lyophilized peptides were reconstituted in distillate H_2O for use in vitro and in vivo. The E7(p) (RAHYNIVTF) was selected because it was identified as an H2-D^b CTL epitope [6]. The H2-K^b-restricted Ova257–264 CTL peptide (SIINFEKL) was used as not related peptide (Ova(p)).

2.4. Adjuvants

VSSP [26] are produced and provided by the Center of Molecular Immunology (CIM, Havana, Cuba) and used at their suggested optimal working concentration of 160 μg/mouse. LPS content in VSSP and OMPC is 30 μg/mg protein and 20–60 μg/mg protein, respectively. Quantification of LPS was performed by measurement of KDO according to the method of Karkhanis et al. [29]. IFA was obtained from Sigma, St. Louis, MO.

2.5. Immunizations

All vaccine formulations were prepared the day before injection, each formulation containing 50 μg of E7(p) in 100 μl was mixed with 160 μg VSSP or emulsified in 100 μl IFA. The total injected volume was 200 μl/mouse. All immunizations were given subcutaneously in the flank, twice at 2-week intervals.

2.6. Implantation and measurement of TC-1 tumor

In preparation for implantation into mice, TC-1 cells were cultured until approximately 70% confluence and harvested with trypsin. TC-1 cells were injected subcutaneously in the leg at various doses, including 5×10^4 and 2×10^5 cells. Starting 7–10 days later and every 3–4 days thereafter, the inoculated area was observed and palpated for the presence of a tumor nodule. Tumor diameters were measured in two orthogonal dimensions using electronic digital callipers (Kell-Strom, Canada). Tumor volumes were calculated from these measurements according to: (length × width^2)/2 [30]. Tumor-bearing mice were killed when moribund, as defined by the HGCULA.

2.7. Cytokine analysis

Spleen cells, harvested from three mice per group, were pooled and cultured in 96-well plates (5×10^5 cells/well) in culture medium containing 10% fetal bovine serum (FBS) and 10 U/ml human interleukin 2 (hIL-2). The spleen cells were sensitized either with or without 1 μg/ml E7(p) for 72 h in triplicate conditions at 37 °C in humidified air containing 7% CO_2. Gamma interferon (IFN-γ) and interleukin 10 (IL-10) were quantified from cell supernatants using a specific ELISA according to the manufacturer's instructions (Quantikine® M murine, R&D Systems, MN, USA). Data were expressed as cytokine released in pg/ml ± S.D. of triplicate ELISA values.

2.8. Enzyme-linked immunospot (ELISPOT) assay

An ELISPOT assay was performed to detect HPV-16 E7-specific $CD8^+$ T cells. A 96-well filtration plate (Millipore, Bedford, MA, USA) was coated with 5 μg/ml rat anti-mouse IFN-γ antibody (clone R4-6A2, Pharmingen, San Diego, CA) in 100 μl of PBS. After overnight incubation at 4 °C, the wells were washed with PBS and blocked with culture medium containing 10% fetal bovine serum (FBS) for 1 h. Mouse $H\text{-}2^b$ target cells (EL4), previously pulsed with 10 μM of the E7(p) and suspended in RPMI 1640 supplemented with 10% of FBS plus hIL-2 (10 units/ml), were used as antigen presenting cells. Unloaded EL4 cells were also included for IFN-γ secreting cells background determination. Serial dilutions of fresh isolated splenocytes from each vaccinated group of mice (10^6, 2×10^5 and 4×10^4 cells per well) and EL4 target cells (10^5 cells per well) were, respectively, loaded to a final volume of 200 μl, and co-incubated by duplicate at 37 °C and 5% CO_2 for 17 h. Later, the plate was washed with PBS containing 0.2% Tween-20 (PBST) and then incubated with 5 μg/ml biotinylated anti-IFN-γ antibody (clone XMG1.2, Pharmingen) in 100 μl of PBS at room temperature for 2 h. After washing with PBST, spot development was performed using HRP–avidin complex (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at room temperature and AEC (Sigma, St. Louis, MO, USA). The spot forming cells (SFC), representing the number of IFN-γ producing cells were counted with a stereoscopic microscope. Values equal to or higher than double of negative control (EL4 without peptide) plus 10 SFC were considered as positive.

2.9. Statistical analysis

The statistical significance of the tumor protection and survival experiments was determined using log-rank test. Differences in tumor volumes among all treatment groups were analyzed by Mann–Whitney *U*-test. For graphic representation of data, *y*-axis error bars indicate the S.D. for each point on the graph.

3. Results

3.1. Prophylactic immunization with E7(p) + VSSP confers protection against recurrent TC-1 tumor challenge

To examine the ability of E7(p) + VSSP immunization to confer protection against in vivo challenge with TC-1, an E7-expressing tumor cell line [28], female C57BL/6 mice were injected subcutaneously on days 7 and 14 with PBS, VSSP, E7(p) + IFA and E7(p) + VSSP. Seven days after the second immunization mice were challenged with 5×10^4 TC-1 cells in the right leg. Fig. 1a shows that TC-1 cell implantation resulted in the appearance of palpable subcutaneous tumors in all mice (10/10) immunized with PBS or VSSP between 14 and 25 days; and all mice (10/10) immunized with E7(p) + IFA developed tumors between 19 and 30 days. In contrast, only 20% (2/10) of mice vaccinated with E7(p) + VSSP showed incidence of tumors at days 22 and 32, respectively, and no progress in the tumor size of these animals was observed. Compared with PBS, VSSP and E7(p) + IFA administrations, E7(p) + VSSP immunization induced statistically significant protection against TC-1 tumor challenge ($P < 0.0001$).

When tumor-free animals from E7(p) + VSSP vaccinated group (five mice) were re-challenged with a larger dose of TC-1 cells (2×10^5 cells) in the left leg, by the end of the 21-day observation period, a similar result was observed with 100% tumor protection (Fig. 1b). As expected, a new cohort of unimmunized mice verified the tumorigenicity of the TC-1 cells used for re-challenge.

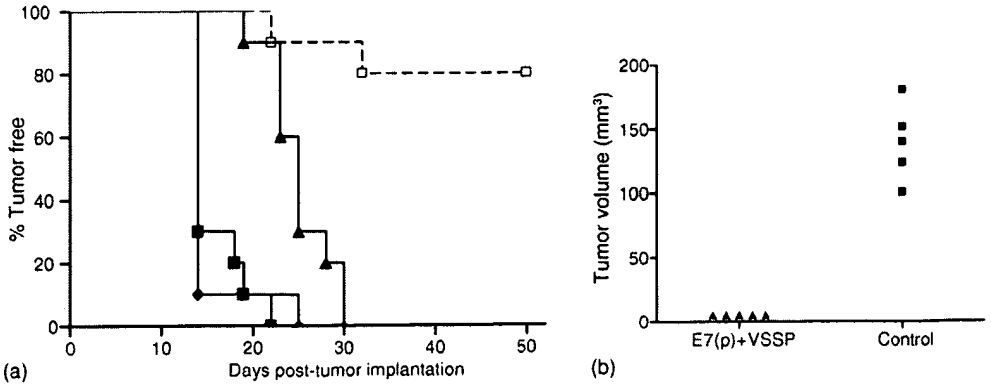

Fig. 1. Prophylactic immunization with E7(p) + VSSP protects against TC-1 tumor challenge and re-challenge. (a) C57BL/6 mice (10 per group) were immunized subcutaneously in the flank with PBS (■), VSSP (♦), E7(p) + IFA (▲), and E7(p) + VSSP (□) and then boosted 14 days later. Seven days after the last boost mice were challenged subcutaneously in the right leg with 5×10^5 TC-1 cells (day 0) and observed for 50 days. Data are presented as percent of tumor incidence per group. (b) On day 50 from previous experiment, five tumor free mice immunized with E7(p) + VSSP were re-challenged with a larger dose of TC-1 cells (2×10^5 cells) in the left flank and observed for additional 21 days. In addition, a new group of untreated mice (control) was challenged with tumor cells on day 50 to verify the tumorigenicity of the TC-1 inoculum. Data are presented as individual tumor volumes from mice.

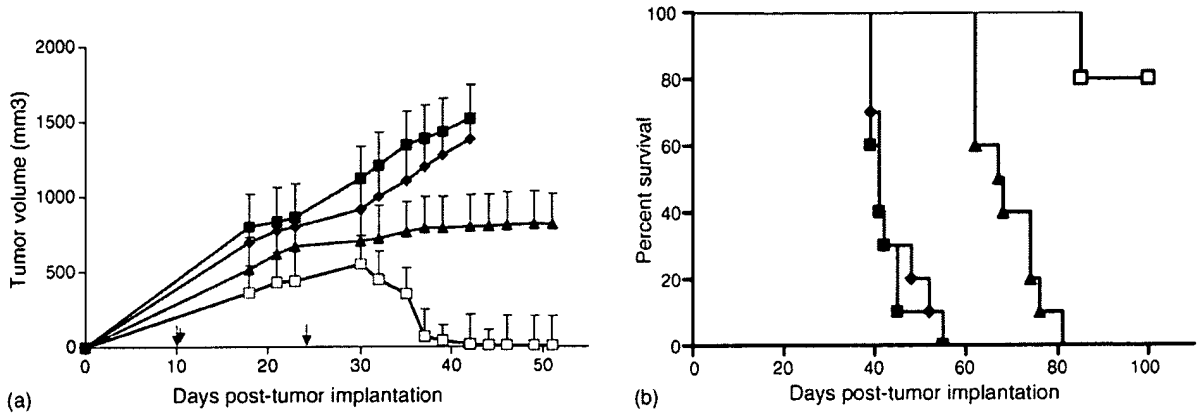

Fig. 2. Therapeutic immunization with E7(p) + VSSP induces TC-1 tumor regression and long-term survival. (a) C57BL/6 mice (10 per group) were injected subcutaneously with 2×10^5 TC-1 cells in the right leg (0). At 10 and 24 days post-implantation, mice were immunized with PBS (■), VSSP (♦), E7(p) + IFA (▲), and E7(p) + VSSP (□) in the flank (arrows). Tumor volumes of subcutaneous nodules were assessed for 50 days post-implantation (as described in Section 2). Data are presented as mean tumor volume (mm^3) ± S.D. in the different groups of mice. (b) Mice were monitored for survival over a 100-day period post-implantation. Mice that became moribund due to tumor burden were killed. Time to death is plotted on a Kaplan–Meier survival curve.

3.2. Therapeutic immunization with E7(p) + VSSP induces TC-1 tumor regression and promotes long-term survival

Given the ability of prophylactic E7(p) + VSSP immunization to confer protection against TC-1 tumor challenge, experiments were performed to determine whether or not E7(p) + VSSP immunization could induce regression of established TC-1 tumors. In these studies, therapy was initiated 10 days post-tumor implantation, when 100% of mice had sizeable subcutaneous tumors. Mice were injected subcutaneously with TC-1 cells (2×10^5 cells) on the right leg (Fig. 2a, day 0) and then 10 and 24 days later they were treated with PBS, VSSP, E7(p) + IFA and E7(p) + VSSP. By the end of the 50-day observation period, when tumor incidence in PBS, VSSP and E7(p) + IFA treated mice was 100%, E7(p) + VSSP therapy led to tumor regression in 100% of mice (Fig. 2a). Compared with PBS, VSSP and E7(p) + IFA treatments, E7(p) + VSSP immunization induced statistically significant regression of TC-1 tumors ($P < 0.0001$).

To determine the effects of E7(p) + VSSP therapy on long-term survival, these animals were followed over a period of 100 days. The Kaplan–Meier survival plot (Fig. 2b) shows that 100% (10/10) of PBS and VSSP-treated animals were moribund by day 55. For E7(p) + IFA-treated group, 100% (10/10) of animals were moribund by day 81. In contrast, survival in the E7(p) + VSSP-treated group was 80% (8/10) over this 100-day period, and it was statistically significant relative to PBS, VSSP and E7(p) + IFA-treated cohorts ($P < 0.0001$).

3.3. Th1 cytokine bias of splenocytes from animals immunized with E7(p) + VSSP

As an initial step in the identification of immune effectors mechanisms associated with tumor rejection, the immune response induced by E7(p) + VSSP immunization was characterized. Mice were immunized with either VSSP, E7(p) + IFA and E7(p) + VSSP and boosted 14 days later. Seven days after the boost, splenocyte cultures were prepared and tested for their capacity to secrete IFN-γ or IL-10 upon re-stimulation with medium, E7(p) or the irrelevant antigen, Ova(p). As shown in Fig. 3, splenocyte cultures from E7(p) + VSSP-immunized mice released high levels of IFN-γ and low levels of IL-10 upon E7(p) restimulation. In contrast, splenocytes from E7(p) + IFA-immunized animals released moderate levels of IFN-γ. Cytokine release appeared to be specific for E7(p), as co-culturing with either medium or Ova(p) (added at the equimolar amount of E7(p)) resulted in release of minimal levels of IFN-γ and IL-10.

3.4. Enhancement of E7-specific $CD8^+$ T cell-mediated immune response by immunization with E7(p) + VSSP with respect to E7(p) + IFA

To compare the E7-specific $CD8^+$ T cell-mediated immunity in different vaccinated groups, the ELISPOT assay was performed. Mice were immunized with either VSSP, E7(p) + IFA and E7(p) + VSSP and boosted 14 days later. Seven days after the boost, splenocyte cultures were prepared. The ELISPOT assay determines the precursor frequencies of activated E7-specific $CD8^+$ T cells [31]. As shown in Fig. 4, the highest ELISPOT numbers were found in mice receiving E7(p) + VSSP. The number of IFN-γ secreting splenocytes in mice immunized with E7(p) + VSSP was approximately nine-fold higher than that of naive animals, whereas in animals immunized with E7(p) + IFA the relation to naive animals was only approximately four-fold higher. The number of IFN-γ secreting splenocytes in mice immunized with E7(p) + VSSP was two-fold higher than that of animals immunized with E7(p) + IFA.

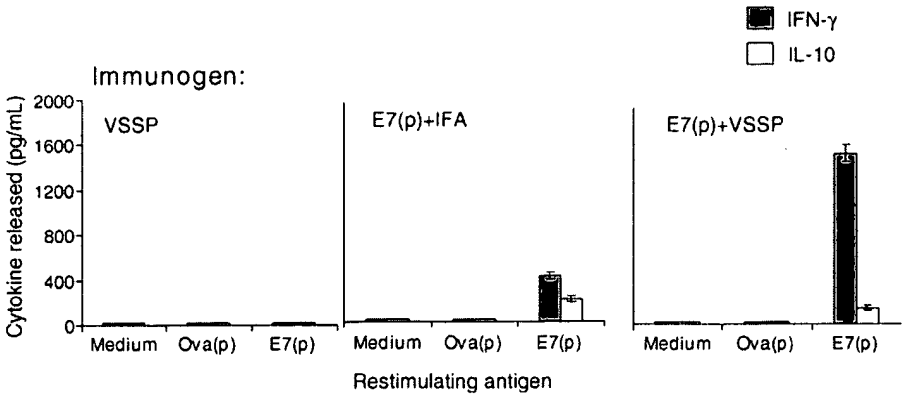

Fig. 3. Splenocytes from E7(p) + VSSP-immunized mice produce IFN-γ upon restimulation. C57BL/6 mice (three per group) were immunized subcutaneously in the flank with either 160 μg VSSP, 50 μg E7(p) + IFA or 50 μg E7(p) + 160 μg VSSP and then boosted 14 days later. Seven days after the boost, pooled splenocyte cultures were prepared and restimulated with medium, 1 μg/ml Ova(p) as irrelevant antigen or 1 μg/ml E7(p), as described in Section 2. IFN-γ (solid columns) and IL-10 (open columns) contents of 72 h culture supernatants were determined by ELISA and expressed as pg/ml ± S.D. of triplicate ELISA values.

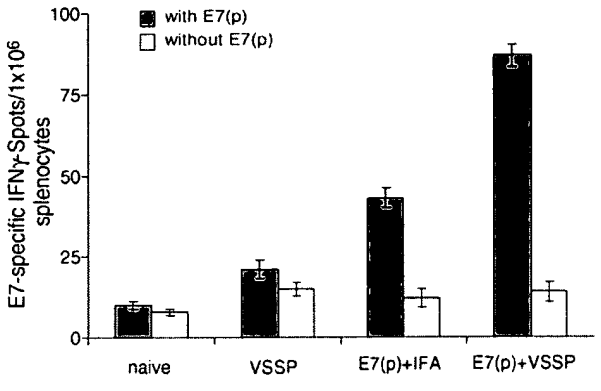

Fig. 4. Demonstration of E7-specific CD8+ T cell precursors in C57BL/6 mice immunized with various immunogens, using ELISPOT assays. Mice (three per group) were vaccinated with VSSP, E7(p) + IFA, E7(p) + VSSP, or were not vaccinated. The splenocytes were harvested 7 days after the last vaccination as described in Section 2. The number of IFN-γ-producing E7-specific T cells precursors was determined in the presence (solid columns) and absence (open columns) of the E7(p). Data are expressed as mean number of SFC per 1×10^6 splenocytes ± S.D.

4. Discussion

The E7 protein of HPV 16 represents an interesting model of a tumor-specific antigen [32] because the expression of this oncogenic early protein is necessary for the maintenance of the transformed state of the HPV-infected cells and is conserved in HPV-induced preneoplastic lesions and cancer [4].

The identification and characterization of CTL epitopes for HPV has promoted the development of peptide vaccines against cervical cancer [6,8]. Several HPV-16 E7-specific CTL epitopes have been characterized for the HLA-A.2 haplotype [8] and have potential clinical applicability. Peptides relevant to other HPV types (HPV-18) [33] and other HLA backgrounds (HLA-B18) are also under investigation [34]. In human studies, CTL responses were observed in some HPV-associated cancer patients after vaccination with lipidate peptides derived from HPV-16 E7 [9] or HPV-18 E6 [35]. In one Phase I/II study, no adverse side effects of peptide-based HPV vaccine were observed in patients [36]. In another study, HPV-16- and HLA-A.2-positive patients with high-grade cervical or vulvar intraepithelial neoplasia were vaccinated with epitopes from E7 HPV16, 12–20 or 86–93; 10 out of 16 patients exhibited measurable enhancement in cytokine release and cytolysis mediated by CTLs derived from PBMCs and some patients had partial clearance of virus and regression of lesions [7]. Both clinical trials have been conducted using IFA [7] and Montanide ISA 51 adjuvant [36].

Many cancer vaccine approaches have managed to induce tumor-specific activation of both CD4 and CD8 T cells. However, these vaccines have generally not been effective enough to produce a real tumor rejection. Particularly, lack of immunogenicity constitutes one of the most significant challenges in peptide vaccine development. Current opinion suggests that the potency of HPV peptide-based vaccines can be further enhanced by the use of stronger-immunostimulatory adjuvants (for a review, see [37]), which may increase the immune response in immuno-compromised individuals. Several studies have demonstrated that peptide antigens must be prepared (formulated) with appropriate adjuvants to facilitate their delivery to DCs and at the same time to activate these antigen-presenting cells to trigger marked T-cell responses.

In this study, we have investigated the antitumor response ability of a vaccine based on the CTL peptide from E7 HPV16 formulated with CIM's proprietary adjuvant, VSSP [26]. This vaccine formulation lead to protection of mice against a lethal dose of HPV-transformed tumor cells, induced the regression of subcutaneous, pre-established E7-expressing TC-1 tumors [28] associated with the induction of an E7-specific, systemic, antitumor immune response, specifically, the release

of IFN-γ by the $CD8^+$ T cells. In addition, in this study we have shown that the antitumor immune response and HPV16 E7-specific $CD8^+$ T-cell response is far more vigorous after vaccination with a E7(p) + VSSP than following vaccination with a E7(p) + IFA.

VSSP belong to the new generation of adjuvants based on pathogen-related molecules identified as "danger" signals that are recognized by the innate immune system [38]. A number of adjuvants evoke strong immune responses, and are widely used in research, but they are unsuitable for human vaccines because of toxic side effects and serious adverse events they can provoke. However, phase I trials of VSSP in melanoma and breast cancer patients have already demonstrated the safety of these preparations [39,40]. Moreover, several trials against meningococcal disease using OMPC of *Neisseria meningitidis* preventive vaccines, an important component of VSSP, were undertaken in the 1980s in Cuba, Norway, Iceland, Brazil and Chile, showing the safety of these preparations in children [41–43].

It has been previously demonstrated that VSSP can stimulate the anti-OVA immune response without the use of oily component, which is associated to the toxicity of emulsified vaccine formulations [27]. Moreover, VSSP have shown the capability to exert an adjuvant effect without any covalent conjugation to the nominal antigen [27]. VSSP may induce potent adjuvant function through DC activation [27] and even though the precise mechanisms are not fully understood, the comparison of VSSP (characterized by a low level of LPS, 3% relative to the total protein) with LPS suggests that, perhaps a pattern recognition molecule in addition to Toll 4 is required for VSSP signalling [27].

Our data suggest that the combination of E7(p) with VSSP by a single mix have preferentially delivered the peptide to DC guaranteeing efficient presentation by MHC class-I molecules to naive T cells, to induce CTL responses and tumor rejection.

Our data are in accordance with data obtained by others in the TC-1 tumor model developed by Wu and colleagues [28], which has been validated for testing novel immunotherapeutics for the treatment of HPV-associated neoplasia. Our results also confirm the potential of the E7 antigen as a target for cancer immunotherapy, which has been previously validated. In contrast to other approaches, E7(p) + VSSP immunotherapy avoids the unwanted side effects of oil-based adjuvant, or the use of live recombinant viral/bacterial vaccines vectors and potentially oncogenic HPV DNA sequences. Moreover, using exact minimal CTL epitope length bypasses the need for antigen-presenting cells to correctly process a whole antigen or large peptides before presenting the T-cell epitopes to the immune system.

In summary, we have demonstrated that a vaccine composed of a minimal CTL peptide mixed in an VSSP adjuvant can generate strong E7-specific antitumor immunity and that this preparation might be successfully used to enhance the presentation of tumor-specific antigens, thereby increasing antigen-specific antitumor immune response. These results suggest that VSSP markedly enhance the number of tumor-specific CTL.

In this work, we have made a proof of concept in mouse using a single CTL peptide mixed with VSSP. In humans, a single epitope-based vaccine will however not likely be effective in all patients since the cellular response must match patient's HLA. However, the use of multiple synthetic CTL epitopes restricted by different HLAs, mixed with VSSP could potentially overcome this restriction, covering ethnic backgrounds, and maintaining an adequate level of cellular immune response.

Since vaccine preparations types used in this study are easy to prepare and safe to administer to humans, our data provide important information for future clinical trials.

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Very small size proteoliposomes derived from *Neisseria meningitidis*: An effective adjuvant for generation of CTL responses to peptide and protein antigens

Circe Mesa *, Joel de León, Luis E. Fernández

Vaccine's Department, Centre of Molecular Immunology, 216 esq 15, Atabey, Playa, CP 16040, C. Habana, Cuba

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Abstract

The development of potent adjuvants, conditioning innate and adaptative immunity, particularly CTL responses, has become currently a hot point in the rational design of vaccines for cancer immunotherapy. We have described a new approach, in which gangliosides are incorporated into vesicles from *Neisseria meningitidis* to form Very Small Size Proteoliposomes (VSSP). VSSP is a good alternative to the existing adjuvants for use in whole cells vaccines since it promotes 80% tumour rejection and growing delay in the CT26 and F3II tumour models respectively. Also VSSP induces activation of CTL responses to co-injected trimmed peptides and soluble proteins. This phenomena is facilitated by the cross-presentation of exogenous antigen and do not need cooperation of CD4 T cells for primary CD8 T cells expansion.

Keywords: CTL; Cross-priming; *Neisseria meningitidis*

1. Introduction

The identification of tumour-associated antigens recognized by CTL at the molecular level suggested that $CD8^+$ T cell responses to tumour cell antigens can be an effector mechanism against cancer [1]. Empirical approaches to induce such responses have met with limited success, but there is great room for improvement and adjuvants have a pivotal role in this task.

Emerging theories about the immune system regulation by the innate immunity have strongly influenced in adjuvant development. Pathogens-related molecules are recognized by receptors of the innate immune system called pattern recognition receptors (PRRs), and are perceived by the innate immune system as molecular signatures of infection switching on the immune system [2]. Based on this theory a new generation of adjuvants has arisen and bacterial products are currently used as effective products [3,4]. However, adjuvants for therapeutic cancer vaccines have to deal with the immune-suppression induced by tumours and this constitute the major challenge faced by tumour immunologist today [5].

In this respect, we have described a new adjuvant that was designed to not only potentiate antigen specific response but also to interfere with the suppression induced by tumours. This formulation is based on natural Outer Membranes Vesicles (nOMV) derived from *Neisseria meningitidis* and the hydrophobic incorporation of the suppressor GM3 ganglioside [6,7] to form Very Small Size Proteoliposomes (VSSP) [8]. This adjuvant combines the stimulatory properties of a pathogen [9] with the promotion of anti-GM3 antibodies [8], which have been suggest diminish immune-suppression induced by this molecule [10,11].

Previous studies with this adjuvant have established that VSSP promote strong antibody and cellular responses to proteins. However, the VSSP adjuvant effect for other accompanying tumour antigen has never been assessed. It has been also demonstrated that the adjuvanticity of this formulation is mediated by proper dendritic cell maturation, with the corresponding IL-12p40/p70 production. Moreover, experiments

* Corresponding author. Tel.: +53 7 2716810; fax: +53 7 272 0644.
E-mail address: circe@ict.cim.sld.cu (C. Mesa).

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with transgenic mice derived T cells showed that VSSP conditioned a Th1 phenotype on stimulated naïve T cells [9]. These results drove our attention to find out whether VSSP could be a vehicle able to activate CTL responses.

In the present, study we evaluated VSSP adjuvant effect for whole tumour cell vaccines showing induction of anti-tumour responses in two different tumour models. Here we also demonstrated that VSSP activate strong specific CTL responses in two different antigen systems: peptides and soluble proteins. We also performed experiments that suggest antigens administered with VSSP gain access to the cytosolic MHC class I presentation pathway and elicit primary CTL response in the absence of CD4 T cell help. This constitutes the first report of activation of CTL by any derivaties of nOMVs from *N. meningitidis*.

2. Materials and methods

2.1. Mice

C57Bl/6 and Balb/c mice were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) and maintained in the animal house of the CIM, Havana, Cuba. All animals were between 6 and 12 weeks of age. Every handling and experiments were performed in accordance with institutional guidelines.

2.2. Cell lines

The murine fibroblast cell line MC57G, the chemically induced murine colon adenocarcinoma tumour cell line CT26 [12], and the GK1.5 hybridoma were purchased from the American Type Culture Collection (Manassas, USA) and cultured in D'MEM (Life technologies Ltd, Paisley, UK). The mammary carcinoma cell line F3II [13] was kindly supply by Dr. D. Alonso, University of Quilmes, Argentina and it is also cultured in D'MEM. B3Z cells (a kind gift of Dr. N. Shastri, University of California, Berkeley), is a T cell hybridoma specific for SIINFEKL in H-2K^b, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promotor and were cultured in RPMI-1640 (Life technologies Ltd, Paisley, UK) [14]. All media were supplemented with 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin.

2.3. Reagents used for stimulation studies

Characterized chicken albumin grade VI (OVA), LPS from *Escherichia coli* strain K-235 and Polyinosinic-Polycytidylic Acid (PIC) was purchased from Sigma Chemical (Dorset, UK). Synthetic peptides used were: $OVA_{257-264}$, SIINFEKL; and human papillomavirus type 16 $E7_{49-57}$, RAHYNIVTF. For in vivo CD4 T cells depletion mAb were isolated by NH_4SO_4 protein precipitation and dialysis of supernatants from GK1.5 hybridoma.

VSSP is produced and provided by the Center of Molecular Immunology (Havana, Cuba) [8]. LPS content of VSSP is 30 μg/mg protein as measured by densitometry analysis after SDS-PAGE and modified silver stains employing periodate oxidation silver staining. Nucleic acid contamination, determined by complete DNase digestion and gel electrophoresis is <5 ng/mg protein, resulting in a maximum potential CpG ODN dose of 1 ng.

2.4. Cellular vaccination and tumour challenge assays

In both, CT26 and F3II tumour models, groups of ten Balb/c mice were immunized subcutaneously (s.c.) in the left flank, with 10^6 irradiated (75 Gy) tumour cells with or without 200 μg of VSSP. Ten days later mice were challenge with a lethal dose (10^5 viable cells) of tumours in the right flank and animals were monitored for 60 days. The largest perpendicular diameters of the resulting tumours were measured with a calliper two times per week, and tumour volume was calculated using the formula $\pi/6 \times \text{length} \times \text{width}^2$. For ethical reasons, animals were sacrificed when the tumour exceeded 10 mm^3 or when the general condition of the animals was affected.

2.5. Immunizations

C57Bl/6 mice were immunized s.c. on days 0, 1, and 8 with 1 mg of OVA, or on days 0 and 14 with 50 μg of SIINFEKL. Both antigen systems were administered in PBS alone or mixed with 120 μg of VSSP. Where indicated, PIC was used as positive control giving one dose of OVA plus 100 μg/mouse of PIC on day 0 and the subsequent 2 days only PIC was administered. On day 10 or 21 for protein and peptide assays respectively, all mice were killed and spleenocytes were colleted for tetramer staining, CD8 T cells specific ELISPOT and ^{51}Cr release assays as indicated. For in vivo CTL assay one single dose of OVA with or without VSSP was injected and 1-week later mice were killed. To deplete CD4 T cells, 1 mg of GK1.5 mAb was given intravenously (i.v.) one day before the immunization and every third day there after.

2.6. FACS analysis

The following reagents were used for flow cytometric analysis: antibody to mouse CD8-Cy5 (YTS.169), phycoerythrin (PE) conjugated anti-mouse CD11c (HL3), anti-mouse CD86/B7.2 (GL1) conjugated to fluorescein isothiocyanate (FITC), (BD Pharmingen, Oxford, UK), and PE conjugated H-2K^b-SIINFEKL tetramer (ProImmune, Oxfrod, UK).

2.7. IFNγ ELISPOT assay

For IFNγ ELISPOT assays, cellulose-ester membrane microplates (Millipore, Hertfordshire, UK) were coated with monoclonal antibody R4-6A2 (BD Pharmingen,

Oxford, UK). Purified CD8 T cells from immune mice (1×10^5 cells/well) were incubated on these plates for 36 h with stimulators consisting of irradiated (30 Gy) syngeneic splenocytes from naïve mice (5×10^5 cells/well), in the presence or absence of 1 μM SIINFEKL. Plates were washed extensively and spots were visualized with biotin-conjugated monoclonal antibody XMG1.2 (BD Pharmingen, Oxford, UK), alkaline phosphatase (AP) conjugated goat anti-biotin (Vector Laboratories, Newcastle, UK) and AP Substrate Kit (Bio-Rad Laboratories, Hempstead, UK), sequentially. Results are expressed as number of spots SIINFEKL specific minus that of non-pulsed stimulators cells.

2.8. ^{51}Cr release CTL assay

For CTL assays, spleen cells from immune mice were cultured in vitro for 5 days with equal numbers of irradiated (30 Gy) syngeneic splenocytes from naïve mice, in the presence of 1 μM SIINFEKL. Viable lymphoblastoid cells were counted by trypan blue exclusion and used as effectors in a standard 6 h ^{51}Cr release assay. Targets consist of radiolabelled, SIINFEKL pulsed (1 μM) MC57G cells and radiolabelled non-peptide pulsed MC57G cells. Results are expressed as the percentage of SIINFEKL specific lysis, representing the percentage of lysis of peptide pulsed MC57G cells minus that of non-pulsed MC57G cells.

2.9. In vivo CTL assay

In vivo cytolytic activity was determined using spleen cells from naïve mice differentially labelled with the fluorescent dye CFSE (Molecular Probes, Paisley, UK) [15]. The cells labelled with $CFSE^{high}$ were used as targets and pulsed with SIINFEKL (1 μM; 90 min at 37 °C, 5% CO2), whereas the cells labelled with $CFSE^{low}$ were leaved unpulsed to serve as the internal control. Peptide-pulsed target cells were extensively washed to remove free peptide and then co-injected intravenously in a 1:1 ratio to previously immunized mice. Sixteen hours later, spleens were removed and the total events corresponding to both fluorescent intensities ($CFSE^{low}$ and $CFSE^{high}$) was determined by flow cytometry. The percentage lysis for each mice was calculated as $100 - (CFSE^{high}/CFSE^{low} \times 100)$.

2.10. B3Z T hybridoma activation assays

For in vitro evaluation of SIINFEKL presentation, bone marrow derived immature murine DCs, prepared as described elsewhere [16], were pulsed with OVA (10 μg/mL) alone or mixed with LPS (0.1 μg/mL) or with VSSP (1 μg/mL). After 8 h, graded numbers of DC were cultured overnight with 5×10^4 B3Z T cell hybridoma in microplates. Supernatants were then discarded and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate (1.5 mg/mL of X-Gal in PBS-NP40 0.25%) (Sigma–Aldrich, Dorset, UK) was added to the remaining cells. The plates were further incubated at 37 °C until positive (blue) signal appeared and B3Z activation was measured by reading plates at 405 nm.

For in vivo evaluation of SIINFEKL presentation, spleens were removed from C57Bl/6 mice 24 h after intravenous injection of 50 μg of OVA in PBS alone or mixed with 200 μg of VSSP. Total splenocytes (5×10^5 cells/well) were incubated overnight with 10^6 B3Z cells/well in 24-well plates. For β-Gal staining B3Z cells, were collected and fixed in PBS-0.5% glutaraldehyde (Sigma, Dorset, UK), and incubated with X-Gal substrate mixture. Cells from each sample were loaded onto a Neubauer counting chamber and viewed under a microscope. The percentage of activated B3Z cells was calculated from the total B3Z counted. B3Z cells alone and DC or splenocytes loaded with SIINFEKL peptide were included as controls.

2.11. Statistical analysis

Equality of variances was analyzed with Bartlett's test and Kolmogorov-Smirnov test was used to analyze normal distribution of data. Differences in F3II tumour growth were evaluated by ANOVA and Tukey test for multiple comparisons. The Mann–Whitney test was used to assess statistical differences between groups from in vitro B3Z assay. Statistical significance of differences between all experimental groups in the rest of the experiments was analyzed by Kruskall Wallis and Student-Newman-Keuls (SNK) tests. Differences in time to progression of CT26 challenged mice was analysed using the Kaplan-Meier method and groups were compared using the Log-Rank test. Data were considered significant when $P \leq 0.05$. All statistical tests were two-sided, and conducted using GraphPad Prism version 4.00 software.

3. Results

3.1. Cellular vaccines adjuvated with VSSP promote antitumour responses

VSSP as adjuvant for irradiated tumour cells as antigen system were assayed in two different tumour models, CT26 and F3II. CT26 cell line is highly immunogenic [17] while F3II is low immunogenic and develops a very aggressive tumour [13]. Mice were vaccinated with CT26 or F3II irradiated tumour cell vaccines followed by a lethal dose of tumour challenge. As shown in Fig. 1A, all control (PBS) mice and 90% of the mice vaccinated with irradiated CT26 along, developed tumours. Noteworthy, a simple mixture of VSSP with the irradiated cells provoked around 80% tumour rejection, remaining tumour-free more than two months after tumour challenge (Log-Rank test $P < 0.05$). This adjuvant effect of VSSP was also observed for the F3II breast cancer cellular vaccine. Although in this poor immunogenic model, the induction of tumour rejection was not detected, either with or without VSSP (data not shown), a clear effect over tumour progression was observed. All tumours rapidly

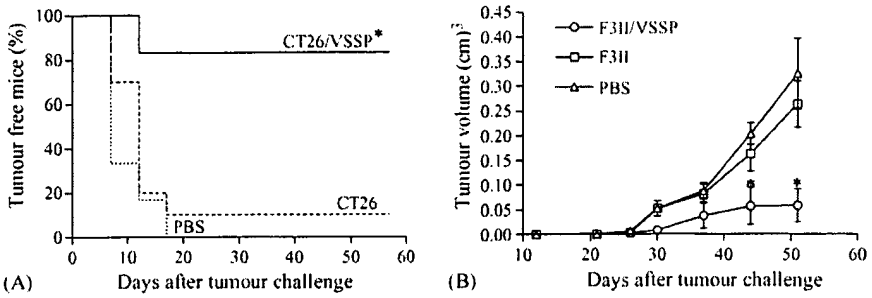

Fig. 1. Antitumour response induce by whole tumour cells adjuvated with VSSP in two different tumour models. Groups of at least six mice were vaccinated with irradiated tumour cells with and without VSSP followed by a lethal dose of tumour challenge. (A) graph represents percent of negative mice after challenge with CT26 tumor cells. (B) Graph represents tumour grow of F3II tumours in every vaccinated groups. (*) Represent statistic significance ($P < 0.05$) according to Log-Rank and Tukey tests for (A) and (B), respectively. Each graph is representative of at least three experiments.

grew in control and irradiated cells treated mice. On the contrary, the addition of VSSP to the cellular vaccine provoked that their sc growing tumours were five times smaller than tumours in the corresponding control animals by day 50 after tumour challenge (Tukey test, $P < 0.05$) (Fig. 1B).

3.2. VSSP promote expansion of OVA specific CD8 T cells

Experiments to address the VSSP capability to induce CD8 T cells activation, highly encouraged by the adjuvant effect of VSSP on both evaluated cellular vaccines, were conducted. Mixtures of OVA with VSSP were injected into C57Bl/6 mice, and the anti-OVA CD8 T cell response measured with H-2K^b-SIINFEKL tetramers. OVA mixed with PIC were employed as positive control. The injection of OVA generated 0.22% of SIINFEKL specific spleenic CD8 T cells while about seven times more specific precursors were generated in mice inoculated with the antigen in the presence of VSSP or PIC (SNK test, $P < 0.05$) (Fig. 2).

3.3. VSSP induce, on CD8 T cells, specific IFNγ secretion and CTL response to a trimmed peptide and to a co-injected protein

To further characterize VSSP capability to induce CTLs activation and because of the pivotal role of IFNγ in the effector functions of these cells against tumours [18], the IFNγ secretion capacity of SIINFEKL specific CD8 T cells was evaluated by ELISPOT (Fig. 3A). Clearly VSSP were necessary to in vivo stimulate OVA specific CD8 T cells to secrete this crucial cytokine (SNK test, $P < 0.05$).

The role of VSSP in the induction of functionally competent specific CD8 T cells was studied with the classical ^{51}Cr release assay. Spleen cells from mice were separated after immunization and restimulated in vitro assessing their ability to kill ^{51}Cr-labeled SIINFEKL-pulsed target cells (Fig. 3B). Just cells isolated from the animals injected with OVA plus VSSP showed a marked cytotoxic activity against the corresponding targets (SNK test, $P < 0.05$).

Due to the poor availability of efficient adjuvants for peptide-based vaccines, VSSP were tested for induction of specific CD8 T cells upon co-immunization with a MHC-I trimmed peptide. A protocol in which mice were subcutaneously inoculated either with SIINFEKL or RAHYNIVTF peptides, mixed with VSSP, on days 0 and 14 was applied. As shown in Fig. 4, SIINFEKL specific CD8 T cells were barely detectable in mice splenocytes after injection of the irrelevant peptide/VSSP mixture. On the contrary a strong response in mice injected with SIINFEKL/VSSP was measured (SNK test, $P < 0.05$). Nearly 0.15% of the total CD8 T cells were specific for SIINFEKL and able to secrete IFNγ.

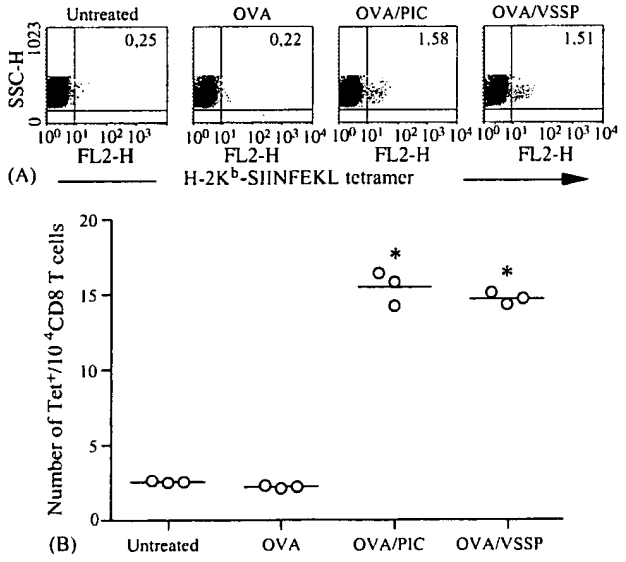

Fig. 2. SIINFEKL specific CD8 T cells expansion after immunization with OVA mixed with VSSP. Groups of three mice were injected with OVA mixed or not with VSSP and SIINFEKL specific CD8 T cells were measured by tetramer staining. PIC was used as positive control (A) Representative dot plots of SIINFEKL specific tetramer staining on the CD8 T cells gated population. Numbers indicate percent of cells on quadrants. One representative mouse per group in shown. (B) Absolute number of SIINFEKL specific CD8 T cells detected in spleens and calculated from the percentage of tetramer positive (Tet$^+$) cells and splenic CD8 T cell counts. (*) Represent statistic significance ($P < 0.05$) according to SNK tests. Data presented are representative of several other experiments.

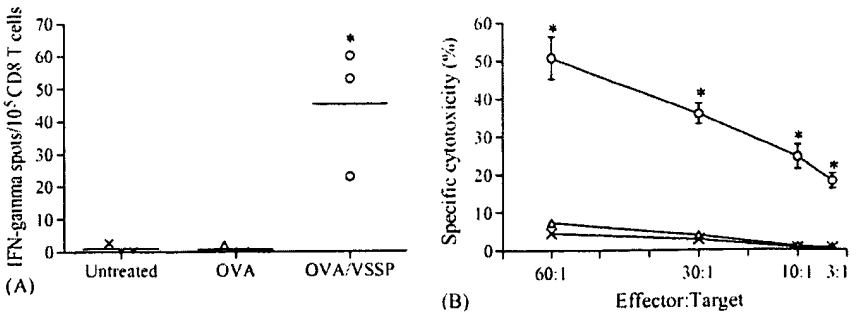

Fig. 3. CTL response induced by co-injection of OVA and VSSP. Groups of three mice were injected with OVA (△), OVA mixed with VSSP (○), or leaved untreated (×) and SIINFEKL specific CTL were measured by (A) IFNγ ELISPOT and data are presented as number of SIINFEKL specific spots calculated from number of detected spots and percentage of CD8 T cells after purification. (B) ^{51}Cr release assay. Data are presented as mean ± S.D. for three mice and (*) represent statistic significance ($P < 0.05$) according to SNK tests. Both graph are representative of at least two separate experiments.

3.4. VSSP facilitate cross-presentation of OVA to specific CD8 T cells.

VSSP capabilities to promote tumour rejection and specific CD8 T cells induction strongly suggest that this adjuvant facilitates antigen presentation on MHC class I molecules. To confirm this point immature murine DCs, pulsed with OVA/VSSP, were cultured with the B3Z T cell hybridoma (which express the β-Gal enzyme upon stimulation with SIINFEKL/K^b complexes). For OVA concentration of 10 μg/mL VSSP were definitely required for the B3Z hybridoma activation. In contrast, no β-Gal activity was observed using OVA mixed with LPS or OVA alone (Fig. 5A) (SNK test, $P < 0.05$).

To test whether VSSP can promote OVA cross-presentation in vivo mixtures of this reference antigen with the small proteoliposomes were sc injected in mice and 18 h later splenocytes were isolated and employed to stimulate B3Z T cells (Fig. 5B). Spleen cells from mice inoculated with OVA/VSSP, but not with OVA alone, induced the expression of β-Gal on B3Z T cells (Mann–Whitney test $P < 0.05$). Noteworthy in vivo and in vitro experiments showed that VSSP induced OVA maximum cross-presentation if we compared levels of the B3Z CD8 T cell hybridoma activation with those measured when the APC where directly loaded with SIINFEKL (Fig. 5).

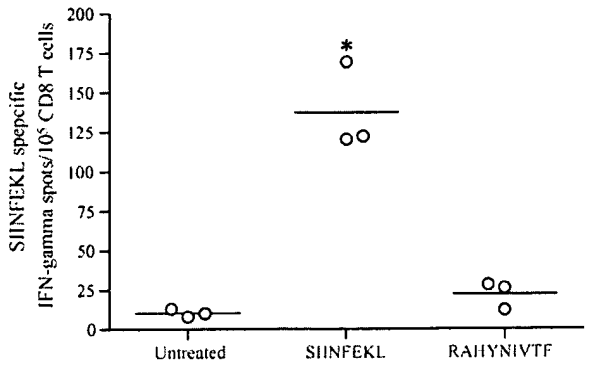

Fig. 4. CD8 T cells specific IFNγ secretion induced by co-injection of SIINFEKL with VSSP. Groups of three mice were injected twice at 14 days intervals with SIINFEKL or RAHYNIVTF in the presence of VSSP and evaluated for the SIINFEKL specific CD8 T cells IFNγ secretion. Data are presented as number of SIINFEKL specific spots calculated from number of detected spots and percentage of CD8 T cells in the spleen from a single experiment representative of three others. (*) Represent statistic significance ($P < 0.05$) according to SNK tests.

3.5. VSSP induce CTL independent of CD4 T cells help

In order to evaluate the VSSP capability to activate CTL responses in the absence of CD4 T cells help, further experiments were conducted in mice treated with antibodies versus CD4 T cells and employing the in vivo CTL assay. One single dose of OVA, with or without VSSP, was sc administered to mice. As shown in Fig. 6 this experiment demonstrated that VSSP were able to induce up to 40% of SIINFEKL positive target cells lysis, even in the absence of CD4 T cells (SNK test, $P < 0.05$).

4. Discussion

Recent advances in our understanding of the immune response are allowing for the logical design of new approaches to cancer immunization. One area of interest is the development of new immune adjuvants to deal, precisely with self and highly tolerated antigens as well as with immunocompromised patients.

The identification of tumour-associated antigens recognized by CTL at the molecular level suggested that $CD8^+$ T-cell responses to tumour cell antigens can be an effector mechanism against cancer [1]. Empirical approaches to induce such responses have met with limited success, but there is great room for improvement and adjuvants have a pivotal role in this task.

VSSP has been previously evaluated in gangliosides positives tumour systems, due to the presence of these molecules into the proteoliposome. In this sense, we have published that VSSP as monotherapy, induce tumour rejection in mice challenged with melanoma B16 cells [19]. In phase I trials

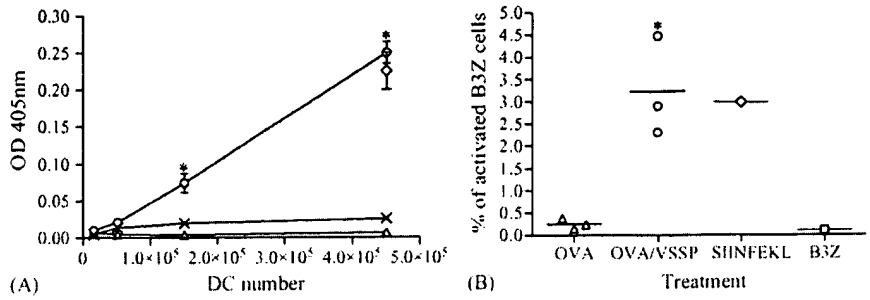

Fig. 5. Activation of SIINFEKL specific B3Z CD8 T cells hybridoma by APC treated with OVA and VSSP in vitro and in vivo. (A) Graded numbers of bmDC pulsed in vitro with OVA (△), OVA plus LPS (×), or OVA plus VSSP (◯) and (B) splenocytes obtained from mice injected with OVA or OVA with VSSP were incubated with B3Z CD8 T cells hybridoma. Activation of B3Z was determined by β-Gal expression detected by incubation with X-Gal substrate. Graph A represent optical density (OD) at 405 nm after subtraction of basal B3Z activation (□). Graph B represent percent of blue cells in a total B3Z population after counting in a Neubauer chamber. In both experiments APC were loaded with SIINFEKL (◇) for maximal occupancy of MHC class I signal. The results are presented as mean ± S.D. and (*) represent statistic significance ($P < 0.05$) according to Mann–Whitney and SNK tests for A and B respectively. This experiment has been repeated three times with similar results.

in melanoma and breast cancer patients, this approach have not only demonstrated the safety and immunogenicity of these preparations [20] but also some encouraging clinical responses.

Here we have demonstrated the adjuvant effect of VSSP in two tumour models using whole cells as antigens. Whole-cell tumour vaccines have been widely investigated because it is assumed that they contain all relevant tumour antigens

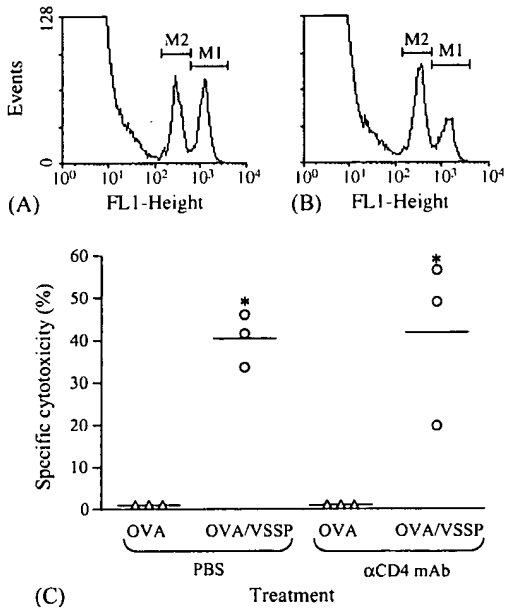

Fig. 6. In vivo CTL induced by VSSP in CD4 T cells depleted mice. Figure shows in vivo killing of SIINFEKL pulsed target cells labeled with $CFSE^{high}$ in normal and CD4 depleted mice after one single dose of (A) OVA alone or (B) OVA mixed with VSSP. Histograms represent one representative mice per immunogen used. (C) Analysis of mean SIINFEKL specific lysis calculated as described in Section 2 (M1 = $CFSE^{high}$ and M2 = $CFSE^{low}$). Groups of treatment are shown in the *x*-axis. (*) Represent statistic significance ($P < 0.05$) according to SNK. This experiment has been repeated many times with similar results.

[21]. These kind of vaccines are usually injected together with powerful immunologic adjuvants, haptens (BCG, diph-theria toxin, dinitrophenyl, keyhole limpet hemocyanin, virus), or both [22], promoting presentation of the tumour antigens in an inflammatory context to attract host APCs. In our studies with the colon carcinoma CT26 cell line, irradiated cells plus VSSP mixtures prevented tumour implantation in 80% of mice. The other tumour cell line evaluated was mammary carcinoma cell line F3II. Contrary to CT26, it is very difficult to accomplish an anti-F3II tumour response [23–25]. However, in our work we have demonstrated that irradiated F3II mixed with VSSP significantly reduced tumour growth. These two events of antitumour response against a solid tumour are generally associated with CTL activation [1]. Moreover, transfer of tumour antigen from the irradiated cell to the host APC, by definition has to be through a cross-presentation mechanism [26]. These results suggested that VSSP facilitate and enhance the natural cross-priming of irradiated cells and induce stimulation of specific CD8 T cells. These results also validate VSSP as a very convenient adjuvant for cellular vaccines.

One approach in the immunotherapy of cancer patients involves vaccination with peptides derived from tumour antigens specifically designed to associate with T cells in the context of MHC class I molecules. Several clinical trials in different tumour types have been conducted utilizing this vaccination strategy [22]. However, it is difficult to draw firm conclusions concerning its efficacy for cancer immunotherapy and improvements, including the addition of stronger adjuvants, most be done. In this paper we have revealed VSSP capacity to stimulate CTL response against a trimmed peptide. We consider very important to point out that VSSP stimulated high quantities of IFNγ production by peptide specific CD8 T cells after injection of a synthetic 8 amino acids peptide only mixed with VSSP. There was no need of covalent conjugation or link between peptide and VSSP. Usually peptide based vaccines need covalent junction with carrier molecules [27] or use oily adjuvants to trap peptides

inside emulsions [28]. This technologic advantage is also valid for the other antigen system evaluated: soluble proteins. We demonstrated by several methods that VSSP stimulate strong CTL response to a co-injected protein. Many well-known adjuvants that have been documented to potentiate CTL responses need covalent conjugation with the antigen. That is the case of an adjuvant also derived from bacteria; Omp-A protein from *Klebsiella pneumoniae*, which primes strong specific CTL responses but needs covalent conjugation to the assayed peptides and proteins [29].

We have recently reported that VSSP interact with dendritic cells to induce their maturation and secretion of important cytokines such as IL-12, which conduced to the subsequent polarization of Th1 CD4 responses [9]. These two phenomenons are in correspondence with the induction of CTL demonstrated here by VSSP. The induction of CTL responses has been already documented for many adjuvants such as CpG [3], HSP [30], kpOmp-A [29], PROVAX [31] and Tomatine [32]. However, this is the first report of CTL stimulation by proteins or any derivate from *N. meningitidis*.

Generation of CTLs to tumours cells requires the presentation of exogenous antigen by MHC class I molecules on professional APCs [33]. Recognition that different antigen-processing pathways control the presentation of antigenic peptides by either MHC class I molecules to $CD8^+$ T cells or MHC class II molecules to $CD4^+$ T cells led to the development of adjuvants that could help target antigens to the desired antigen processing pathway. Adjuvants that have been shown to facilitate this phenomenon of cross-presentation include among others HSP [34] and CpG [35], but others such as LPS fails to do so. In this work we showed that VSSP facilitate presentation of exogenous protein derived peptides and present them to a specific CD8 T cell hybridoma, while LPS, as it is widely accepted, did not. This result also corroborates the previous result in the C3H/HeJ mice, which demonstrated that VSSP adjuvant effects were not only due to the presence of LPS in its composition [9].

Over the years, many types of stimuli have been tested to determine whether CD4 T cell help is required for the generation of effective CTL immunity. These studies have reported the existence of both helper dependent and independent CTL responses [36]. However, during last years this concept has been review and an alternative explanation for CD4 T cell help dependence has been postulated. These new theory suggested that all responses were helper independent in the primary phase but depended on help for secondary expansion [37]. While this model is becoming widely accepted few experiments showing reduction of primary expansion of naive CD8 T cells in the absence of help, have overlapped both concepts and restricted the original model to the primary phase. For example, primary OVA-specific CTL activity could not be detected in class II-deficient mice primed with OVA-coated spleen and LPS, although efficient responses were seen in wild-type mice [36]. In this work we have reported that primary specific CD8 T cell response induced with OVA plus VSSP remained unaltered on CD4 T cell depleted mice. This result includes VSSP in the group of adjuvants that are able to elicit CTL responses to exogenous antigen in the absence of CD4 T cell help.

This finding also constitute another evidence that LPS is not responsible of every VSSP adjuvants properties but we do not discard the fact that combination of LPS, outer membrane proteins of *N. meningitidis* or included gangliosides, could be advantageous over effects of individual components. Particularly the presence of GM3 in our preparation could not only play a role in its physicochemical characteristics but also a crucial biological function cannot be discarded and is currently under investigation. Gangliosides are important molecules on TCR signalling [38] and are closely involved with the evasion mechanism of many tumours [39], suggesting that its presence could make the difference of VSSP, over other adjuvants, as adjuvant for therapeutic cancer vaccines.

In this paper, we have amplified the concept that VSSP provide danger signals to induce DC activation and Th1 polarization [9] to the fact that this VSSP signals activates and expands antigen specific CD8 T cells. This phenomena is facilitated by the cross-presentation of exogenous antigen and do need cooperation of CD4 T cells for primary expansion. Therefore, VSSP could be a good alternative to the existing adjuvants for use in future vaccines and can be effectively used as an adjuvant of whole cells, peptides and soluble proteins.

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